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(57) Abstract

Genes which encode ACC synthase are identified for the geranium and rose plants, specifically *Pelargonium X hortorum* cv sincerity and *Rosa* (cardinal red). These genes are shown as modified to achieve a transgenic plant which resists wilting and the like as a result of reduced ethylene production. This alteration is reproduced by the transformed plant. Isolation of high quality mRNA is achieved through the use and adaptation of a 2–butoxyethanol precipitation technique using a large amount of initial tissue in order to achieve critical mass for precipitation.

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1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE GENES FROM *PELARGONIUM* AND *ROSA* TO CONTROL ETHYLENE LEVELS IN GERANIUMS AND ROSES

TECHNICAL FIELD

This invention relates to the field of compositions and methods for inhibiting the enzyme 1-aminocyclopropane-1-carboxylate (ACC) synthase in both geranium and rose thereby prolonging the shelf-life of cut flowers as well as reducing leaf yellowing and petal abscission during shipping and storage.

BACKGROUND

A variety of factors cause wilting and natural abscission in flowers, particularly after a cutting of the plant or when flowers have been removed from the plant. Such factors include increased oxygen levels, wounding, chemical stress, and the plant's own production of ethylene. Of these factors, the plant's production of ethylene, has been shown to play a key role in natural senescence, the degenerative process which generally leads to controlled cell death in plants, but also in the degradation of flowers after they have been cut.

Ethylene, in all higher plants, is important to plant growth and development from seed germination, seedling growth to flowering and senescence (Abeles, F.B. *et al.* (1992), In: *Ethylene in Plant Biology*. Eds. Abeles, F.B. *et al.*, Academic Press, New York, pp 285-291 and 1-13; Yang, S.F. *et al.* (1984), *Annu. Rev Plant Physiol*:35, 155-189). Ethylene production in plants can also be associated with trauma induced by mechanical wounding, chemicals, stress (such as produced by temperature and water amount variations), and by disease. Hormones can also stimulate ethylene production. Such ethylene, also sometimes called "stress ethylene", can be an important factor in storage effectiveness for plants. Moreover, exposure of plant tissue to a small amount of ethylene often may be associated with increased production of ethylene by other adjacent plants. This autocatalytic effect may be often associated with losses in

marketability of plant material during storage and transportation (Abeles *et al.*, *supra*; Yang *et al.*, *supra*).

The ethylene biosynthetic pathway in plants was established by Adams and Yang (Adams D.O., *et al.*, (1979) *Proc. Nat'l Acad Sci USA 76*: 170-174)). The first step involves the formation of S-adenosyl-L-methionine (AdoMet) from methionine by S-adenosyl-L-methionine synthetase. AdoMet is then converted into 1-aminocyclopropane-1-carboxylate (ACC), the direct precursor of ethylene in higher plants. This conversion is catalyzed by ACC synthase (S-adenosyl-L-methionine methyl thioadenosine-lyase, EC4.4.1.14), the rate limiting step in the ethylene biosynthetic pathway. (*See also* Kionka C., *et al.*, (1984) Planta 162:226-235; Amrhein N. *et al.*, (1981) *Naturwissenschaften 68*: 619-620; Hoffman N.E., *et al.*, (1982) Biochem Biophys Res Commun 104 765-770).

Knowledge of the biosynthetic pathway for ethylene formation has been fundamental in developing strategies for inhibiting ethylene production in plants. One approach has been to use chemical inhibitors to inhibit the synthesis or activity of ethylene, two of the most common being aminoethoxyvinylglycine and aminooxyacetic acid (Rando, R.R., 1974, Science, 185, 320-324 and in Ethylene in Plant Biology, (Abeles, F.B., *et al.*, eds. Academic Press, p. 285)). However, chemical methods find limited use because such methods are expensive and the beneficial effect they provide is generally only short-lived.

A second approach has been to overexpress ACC deaminase, an enzyme which metabolizes ACC, thereby eliminating an intermediate in the biosynthesis of ethylene (Klee, *et al.*, (1991) Cell 3: 1187-1193) (*See also* Theologis, A., *et al.* (1993), Cellular and Molecular Aspects of the Plant Hormone Ethylene, p. 19-23). Because ACC deaminase is a bacterial enzyme, it is heterologous, and thus, external to the plant. Thus, it is unlikely that this approach will yield a modification that will be stable from generation to generation.

Yet another approach involves attempts to genetically inhibit the production of the enzymes involved in the biosynthesis of ethylene or to inhibit the biosynthesis of the

enzymes directly. This approach has the advantage of not only altering the way the plant itself functions irrespective of external factors but also of presenting a system which reproduces itself, that is, the altered plant's progeny will have the same altered properties for generations to come.

Initial efforts to better understand the enzymes which catalyze the reactions in the biosynthesis of ethylene have involved the identification and characterization of the genes encoding for AdoMet synthetase, ACC synthase, and ACC oxidase (*See also* Kende H.., 1993, Annu Rev Plant Physiol Mol Biol 44:283-307). Some of the genes encoding for ACC synthase have been identified for a number of plants. For instance, ACC synthase sequences have been identified for zucchini (Sato T., *et al.*, (1989) *Proc. Natl Acad Sci USA 86*:6621-6625), winter squash (Nakajima, N., *et al.*, (1990) *Plant Cell Physiol 31*:1021-1029), tomato (Van Der Straeten, D., *et al.*, (1990) *Proc Natl Acad Sci USA 87*:4859-4863); (Rottmann, W.H., *et al.*, (1991) *J Mol Biol 222*:937-961), apple (Dong, J.G., *et al.*, (1991) Planta 185:38-45), mung bean (Botella, J.R., *et al.*, (1992a) *Plant Mol Biol 20*:425-436; Botella, J.R., *et al.*, (1993) Gene 123: 249-253; Botella, J.R., *et al.*, (1992b) *Plant Mol Biol 18*: 793-797); Kim, W.T., *et al.*, (1992) *Plant Physiol 98*:465-471), carnation (Park, K.Y., *et al.*, (1992) *Plant Mol. Biol.*, 18, 377-386), *Arabidopsis thaliana* (Liang, X., *et al.*, (1992) *Proc Natl Acad Sci USA 89*:11046-11050; Van Der Straeten, D., *et al.*, (1992) Proc Natl Acad Sci USA 89:9969-9973), tobacco (Bailey, B.A., *et al.*, (1992) *Plant Physiol 100*: 1615-1616), rice (Zarembinski, T.I., *et al.*, (1993) *Mol Biol Cell 4*: 363-373), mustard (Wen, C.M., *et al.*, (1993) Plant Physiol 103:1019-1020), orchid (O'Neill, S.D., *et al.*, (1993) Plant Cell 5: 419-432), broccoli (Pogson, B.J., *et al.*, (1995) *Plant Physiol 108*:651-657), and potato (Schlagnhaufer, C.D., *et al.* (1995) *Plant Mol. Biol. 28*:93-103).

That ACC synthase is involved in the ethylene pathway is confirmed by the fact that increased levels of ACC synthase mRNA correlate with an increased activity of ACC synthase in plants during fruit ripening and flower senescence. Similar correlation is also observed in response to exogenous signals caused either by wounding or due to treatment with hormones such as auxin, cytokinin and ethylene. Interestingly, the expression of

different classes of ACC synthase occurs from a variety of signals in a many plants, e.g. four different ACC synthase genes have been shown to be differentially expressed in tomato fruit, cell cultures, and hypocotyls during ripening, wounding, and auxin treatment (Olson, D.C., et al (1991) Proc. Natl. Acad. Sci. USA 88:5340-5344; and Yip, W.K., (1992) Proc. Natl. Acad. Sci. USA 89:2475-2479). Differential expression of two ACC synthase genes has also been observed in winter squash during wounding or by auxin (Nakajima, *et al.* (1990) Plant Cell Physiol, 31; 1021-29 and (1991) Plant Cell Physiol, 32; 1153-63). Similar differential regulation of expression ACC synthase genes takes place in carnation flowers by wounding or during senescence (Park, K.Y., *et al.*, (1992) *Plant Mol. Biol.*, 18, 377-386). The evolution of ACC synthase genes into a multigene family that responds differentially during plant development or in response to stimuli external to the plant (Rottmann, W.H., *et al.*, (1991) *J Mol Biol 222*:937-961) may be a reflection of the importance of ethylene in plants. (*See also* Slater, A., *et al.*, (1985) Plant Mol Biol 5:137-147). (Smith, C.J.S., *et al.*, (1986) Planta 168; 94-100 and Smith, C.J.S., *et al.* (1988) Nature 334;724-26). (Hamilton, A.J., *et al.*, (1990) Nature 346:284-286; Köck, M., *et al.*, (1991) Plant Mol Biol 17:141-142).

The discovery of the foregoing and of other properties has lead to an understanding that it may be desirable to attempt to genetically alter the production of ethylene in plants. This approach, however, may be considered in some ways delicate. Elimination of ethylene is not a desired result as in many instances it will kill the plant. Modulation of ethylene -- at the appropriate times -- is the critical goal, not elimination of it entirely. This has been attempted at at least two points in the pathway: the production of ACC by ACC synthase, and the oxidation of ACC by a different enzyme, ACC oxidase. Because the ACC synthase approach can permit stable modulation and not only total elimination of ethylene, it is a preferred technique. To date, however, successful reduction of the production of ethylene through an alteration at the ACC synthase step in the pathway has only been accomplished in one plant, tomato(Oeller, *et al.* (1991) *Science 254*:437-39). In spite of the seemingly simple conceptual nature of this goal, the actual accomplishment of an alteration of the ethylene biosynthetic pathway through the ACC synthase technique has remained elusive. This is particularly true for

the geranium and rose plants, perhaps due to the fact that the identification of full length genes can be difficult for plants. As discussed later, this may, in part, be due to the fact that isolation of full length or high quality RNA has been deemed "notoriously difficult" for plants. (John, M.E., Nucleic Acids Research 20:2381, 1992, and Logemann, J. et al, Anal Biochem 163, 16-20, 1987).

Efforts by others highlight some of the difficulty involved. Recently, Arteca's laboratory (Wang, T.W. *et al.*, (1995) *Plant Physiol. 109*:627-636) studied two cDNA molecules encoding ACC synthase from a white flower variety of a flowering geranium plant (*Pelargonium x hortorum* cv Snow Mass Leaves). As their publication explained (perhaps after the fact), these researchers tried to identify and characterize two clones, GAC-1 and GAC-2. In spite of their efforts, they were only able to completely identify one of those cDNA gene sequences, GAC-1. Their study examined the expression of these ACC synthase genes in different plant parts of the geranium and in response to stress induced by osmotic changes (sorbitol) or metal ions ($CuCl_2$). It also evaluated the effects of ethylene on auxin 2,4-D induction in geranium leaves. The study indicated that GAC-1 expression was induced only by stress, whereas expression of GAC-2 appeared to be developmentally regulated. Furthermore, these authors speculated about possible future "transfer of antisense GAC-1, GAC-2 ... into *Pelargonium* tissues through the *Agrobacterium* transformation or particle bombardment." This confirms a desire in the art for an ACC synthase approach to altering ethylene production in such plants. In spite of this desire, however, the isolation and identification of some, if not all, the ACC synthase gene sequences -- for geranium remained elusive. In similar fashion, rose as well has remained elusive.

Although several plant ACC synthase genes have been identified and sequenced, the current invention describes ACC synthase gene sequences which were previously unknown and which are not believed to have been easily discoverable. As mentioned, one factor which may have militated against an expectation of successfully cloning a plant gene is the particular difficulty in obtaining high-quality and full-length RNA from plants. Indeed, this process has been characterized as "notoriously difficult" by at least

more than one practitioner of the art (John, M.E., *Nucleic Acids Res. 20*:2381, 1992 and Logemann, J., et al, *Anal Biochem 163*, 16-20, 1987)). While this proved to be true for the present inventor, these difficulties were overcome by assessing a new approach to the RNA isolation process. The current inventor, after finding traditional RNA isolation methods to be ineffective, was forced to develop a non-traditional approach described herein. Basically, even though those of ordinary skill in the art had long desired to identify some gene to manipulate to alter the production of ethylene in some plants, in this case, they failed to realize that the problem lay in the need for a better isolation process. Even though the implementing technology for this process had long been available, those in the art apparently failed to realize how to use that technology to achieve the results now described. To some extent they simply may not have defined the problem, preventing the achievement the goals sought. Their efforts may properly be characterized as having taught away from the direction taken by the present inventor and, thus, the results achieved here should be considered unexpected.

Difficulties in isolating full-length mRNA in the specific case of geranium are also further reflected by the fact that one of the sequences isolated by the current inventor (clone pPHSacc49), though it may bear some similarity to portions of the clone termed GAC-2 by Wang *et al., supra,* (which, in any case, may have been discovered after the making of the present invention) is actually considerably longer than GAC-2. This highlights the difficulty in successfully isolating a full-length mRNA molecule using standard RNA isolation procedures in certain plant materials. Furthermore, the current inventor has isolated a third novel full-length clone (pPHSacc44). Moreover, the high quality RNA (as defined below) isolated by the current inventor is further evidenced by the fact that full length cDNA clones were obtained, and all of them could be successfully expressed in an *in vitro* expression system. In each case, full length ACC synthase (enzyme) protein is synthesized *in vitro*. In contrast, even later publications by Arteca's group do not describe the actual *in vitro* expression of any of the isolated DNA clones. In fact the cDNA for the GAC-2 gene was never isolated. Rather, only a partial sequence was merely deduced from the sequence of genomic clones.

This is significant because it highlights the difficulty in isolating and thereby identifying full length ACC synthase genes. Those of ordinary skill in the art had faced the same challenge. Derivation of DNA encoding ACC synthase from a genomic clone rarely is successful, and therefore, simply would not provide a reasonable expectation of success to one of ordinary skill. Only by utilizing a new and different approach did the present invention successfully identify not only one but several full length ACC synthase gene sequences from the geranium plant. The same technique applies to the identification of the ACC synthase gene sequence from the rose plant. Basically, it was this high quality library containing full length cDNA clones which allowed the present inventor to successfully achieve direct cloning of ACC synthase cDNA. The prior art did not discover these sequences because it could not have: the genes did not exist in the available libraries. It was this new approach which overcame the problems faced, but not solved, by others and resulted in the extraordinary successes described herein. The extraordinary success of the present invention -- a nearly one hundred fold increase in positive identifications -- is a consequence of the new technique for RNA isolation and cDNA identification, and not the result of analogous knowledge gained from the efforts of others. Mere comparison to other genes in the same or different plants did not and could not have yielded the successes described here. The existence of the cDNAs of interest in the library was the governing factor. Thus, even with a viable identification process, successful identification of the several geranium ACC synthase genes and the rose ACC synthase gene, let alone the actual alteration of the plants thenselves by means of this knowledge, would not have been likely.

Additionally, it should be understood that knowledge of the full length sequence of a gene from other plants simply does not necessarily lead one to the sequences of the homologous genes in the geranium or such a gene in the rose plants. First, as mentioned earlier, the genes encoding ACC synthase have evolved into a multigene system in some cases. There appears to be no single gene, but rather a family of genes in most cases. Thus, knowledge of one gene in one plant species is not certain to lead to one (or several) homologous or analogous genes in another plant species. Second, because known ACC synthase genes are typically so diverse in their nucleotide sequences, knowledge of one

would not lead a person of ordinary skill in the art to an expectation of success in isolating the ACC synthase gene from either geranium or rose.

Antisense technology is a well known approach to creating a plant that produces less of a selected protein. Through this technology, a plant is altered by introducing a foreign DNA sequence that encodes an mRNA product complementary to part or all of the plant's "sense" mRNA encoding the protein. The presence of antisense RNA inhibits RNA function within a cell (and whole organism). Antisense RNA can bind in a highly specific manner to its complementary sense RNA resulting in blockade in processing and/or translation of the sense mRNA. Antisense RNA may also disrupt interactions between sense mRNA and sequence-specific RNA binding proteins. Antisense technology may be employed to inhibit the synthesis of an enzyme involved in ethylene biosynthesis. The genes identified by the current inventor and disclosed herein have been used for the conception and implementation of antisense sequences specific for ACC synthase mRNA. Introduction of DNA encoding such antisense RNA sequences into a geranium or rose plant is highly probable to result in a plant which stably produces less ethylene.

The incorporation of antisense RNA in plants as a means to inhibit the synthesis of enzymes has been described by various investigators. Rothstein, *et al.* (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*: 8439, found that antisense RNA inhibited nopaline synthase (*nos*) in tobacco. Smith, C.J.S., *et al.* (1988) *Nature 334*: 724, reported that antisense RNA inhibited polygalacturonase in tomato. Others have used antisense RNA to inhibit the synthesis of enzymes involved in ethylene formation. Oeller, P.W., et al., (1991) *Science 254:* 437-439, expressed RNA antisense to ACC synthase in tomato plants. Others have expressed antisense RNA to a different ethylene forming enzyme (EFE), ACC oxidase, in carnation and tomato (Michael, M.Z., *et al.*, 1993, In: Pech, J.C., *et al.*, eds., Cellular and Molecular Aspects of the Plant Hormone Ethylene (Kluwer Academic Publishers, pp. 298-302); Hamilton, A.J., *et al.* (1990) *Nature 346*: 284-287; Gray, *et al.* (1993), in Pech, J.C., *et al.*, *supra,* pp. 82-89; Murray, A.J., *et al.* (1993) in Pech, J.C., *et al.*, *supra,*, pp. 327-328). The above work with antisense RNA may also be

applicable to efforts to stably incorporate the sequences identified by the current inventor and their antisense sequences into both geranium and rose plants. Similarly, the success in expressing antisense RNA for ACC synthase in tomato plants may also be applicable (Oeller, *et al.*, *supra*). It is noteworthy, and perhaps surprising, that neither of the foregoing disclosures have led to the long sought goal of stably altering ethylene production in either geranium or rose plants. Hence, neither an altered geranium plant or altered rose plant expressing reduced levels of ethylene has been described. The incorporation of ACC synthase antisense DNA into either a geranium or a rose plant has remained elusive because the complete ACC gene sequences were not available prior to the present invention. The discoveries disclosed herein enable the production of an appropriately altered geranium plant and an appropriately altered rose plant each of which will express ACC synthase antisense sequences and stably produce reduced levels of ethylene.

DISCLOSURE OF THE INVENTION

This invention is based on the discovery and cloning of multiple 1-amino cyclopropane-1-carboxylate (ACC) synthase cDNA molecules. In geranium, there are three molecules which represent three ACC synthase genes from *Pelargonium hortorum cv Sincerity* (red flowered cultivar of the geranium genus). In rose, there is one molecule which represents the ACC synthase genes from *Rosa* (actually the cardinal red rose cultivar of the rosa genus). The nucleotide sequence and corresponding amino acid sequence for each of these genes is disclosed herein. Importantly, this is believed the first report of the full-length sequence for each gene, evidenced by the ability of the cDNAs to be expressed in an expression system. Moreover, clone pPHSacc44 for geranium is shown to contain unique and important regulatory sequences.

The invention provides a method for genetic modification of geranium and rose plants to control their levels of ethylene. The newly discovered DNA sequences, fragments thereof, or combinations of such sequences or fragments, are introduced into a plant cell in reverse orientation to inhibit expression of ACC synthase, thereby reducing

the levels of endogenous ethylene.

Using the above methods or plant-specific variants of them, transgenic plants are to be developed and monitored for growth and development. Those plants exhibiting prolonged shelf-life with respect to plant growth, flowering, and/or reduced yellowing of leaves due to reduction in levels of ethylene are to be selected and propagated as premier products with improved properties including reduced leaf yellowing and petal abscission during shipping and storage.

The present invention is directed to an isolated DNA molecule encoding an ACC synthase enzyme of geranium which DNA molecule hybridizes with pPHSacc41 (SEQ ID NO:1), pPHSacc44 (SEQ ID NO:2), or pPHSacc49 (SEQ ID NO:3), or a functional derivative of the DNA molecule which hybridizes with SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3 and to an isolated DNA molecule encoding an ACC synthase enzyme of rose which DNA molecule hybridizes with pRoseKacc7 (SEQ ID NO:14) or a functional derivative of the DNA molecule which hybridizes with SEQ ID NO:14.

The isolated DNA molecule is preferably one with substantial sequence homology with a molecule selected from, for geranium, the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3, and for rose, the molecule set out in SEQ ID NO:14. In one embodiment for geranium, the isolated DNA molecule is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3. In one embodiment for rose, the isolated DNA molecule is that of SEQ ID NO:14.

In another embodiment for both geranium and rose, the present invention provides an isolated protein encoded by a DNA molecule as described above, or a functional derivative thereof. For geranium, a preferred protein has an amino acid sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, or is a functional derivative thereof. For rose, a preferred protein has an amino acid sequence of SEQ ID NO:15 or is a functional derivative thereof.

Also provided herein is an antisense oligonucleotide or polynucleotide encoding an RNA molecule which is complementary to at least a portion of an RNA transcript of the DNA molecules described above, which RNA molecule hybridizes with the RNA

transcript such that expression of the ACC synthase enzyme is altered.

The above antisense oligonucleotide or polynucleotide molecule can be full length or preferably has between six -- or ten, twenty, or fifty -- and 100 nucleotides.

For geranium, the antisense oligonucleotide or polynucleotide may be complementary to at least a portion of one strand of the nucleotide sequence SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3, or may be complementary to at least a portion of an RNA sequence encoded by SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3. In one embodiment, the antisense oligonucleotide is complementary to at least a part of a 5' non-coding portion of one strand of the nucleotide sequence SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3. Similarly, for rose, the antisense oligonucleotide or polynucleotide may be complementary to at least a portion of one strand of the nucleotide sequence SEQ ID NO:14 or may be complementary to at least a portion of an RNA sequence encoded by SEQ ID NO:14. In one embodiment, the antisense oligonucleotide is complementary to at least a part of a 5' non-coding portion of one strand of the nucleotide sequence SEQ ID NO:14.

For geranium, an antisense oligonucleotide as described above may be complementary to at least a part of the nucleotide sequence SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3, which part is, for example, from nucleotides 1-50; nucleotides 51-100; nucleotides 101-150; nucleotides 151-200; nucleotides 201-250; nucleotides 251-300; 301-350; 351-400; 401-450; or 451-500; or any other such contiguous group up to nucleotide 500, 1000, or to the end of the gene. Similarly, for rose, an antisense oligonucleotide as described above may be complementary to at least a part of the nucleotide sequence SEQ ID NO:14, which part is, for example, from nucleotides 1-50; nucleotides 51-100; nucleotides 101-150; nucleotides 151-200; nucleotides 201-250; nucleotides 251-300; 301-350; 351-400; 401-450; or 451-500; or any other such contiguous group up to nucleotide 500, 1000, or even to the end of the gene.

This invention is further directed to a vector useful for transformation of either a geranium or a rose plant cell, comprising:

(a) an antisense oligonucleotide or polynucleotide as described above ;

(b) regulatory sequences required for expression of the oligonucleotide or polynucleotide in the cell.

The regulatory sequences comprise a promoter active in the cell, which may be an inducible promoter or preferably, a constitutive promoter. The vector preferably further comprise a polyadenylation signal.

In the above vector the promoter is preferably a heterologous promoter such as a viral promoter. A preferred viral promoter is the CaMV 35S promoter or a promoter homologous to CaMV35S.

In other embodiments, the promoter is selected from the group consisting of the SSU gene promoter, ribulose bisphosphate carboxylase promoter, chlorophyll a/b binding protein promoter, potato ST-LS1 gene promoter, soybean heat shock protein hsp17.5-E promoter, soybean heat shock protein hsp17.3-B promoter, phenylalanine ammonia-lyase promoter, petunia 5-enolpyruvylshikimate-3-phosphate synthase gene promoter, *Rhizobium meliloti* FIXD gene promoter and nopaline synthase promoter.

Also provided is both a geranium cell and a rose cell, each transformed with a vector as described above, a plantlet or mature geranium or rose plant generated from such a cell, or a plant part from such plants.

The present invention is further directed to a method to alter expression of an ACC synthase enzyme in both a geranium cell, plant or a cutting thereof and a rose cell, plant or a cutting thereof, comprising

(a) transforming either a geranium or rose cell or plant with a vector according to any of the prior directions; and

(b) allowing the antisense oligonucleotide or polynucleotide to be expressed and to hybridize with nucleic acid molecules in the cell, plant or cutting which encode the ACC synthase enzyme.

Also provided is a method of producing both a geranium and a rose plant having reduced ethylene production compared to an unmodified plant, comprising the steps of:

(a) transforming either a geranium or a rose plant with a vector as above;

(b) allowing the plant to grow to at least a plantlet stage;

(c) testing the plant for ACC synthase enzymatic activity or ethylene production; and

(d) selecting a plant having altered ACC synthase activity and/or altered ethylene production compared to an unmodified geranium or rose plant

A geranium or rose plant produced as above, or progeny, hybrids, clones or plants parts thereof, preferably exhibits reduced ACC synthase expression and reduced ethylene production.

In another embodiment, the invention is directed to a method for producing either a geranium or a rose variety (or line), characterized by reduced expression or activity of an ACC synthase enzyme and reduced ethylene production compared to an unmodified geranium or rose variety, comprising producing a geranium or rose plant as above and selfing the plant to generate the variety.

Also provided is a method for producing a variant plant of a non-geranium or non-rose species, an ACC synthase gene of which is homologous to either a geranium or rose ACC synthase gene, in which variant plant the ACC synthase expression is altered in comparison to an unmodified plant of the species, comprising

(a) identifying and isolating an ACC synthase gene of the species by hybridization with a sense DNA molecule as described above

(b) constructing a vector which comprises an antisense DNA sequence encoding at least a part of the gene identified in step (a) in an antisense orientation such that

(i) an RNA transcript of the antisense DNA sequence is complementary to the part of the gene, and

(ii) expression of the antisense DNA sequence alters expression of the ACC synthase gene;

(c) transforming a cell of a plant of the species with the vector of step (b) to generate a transformed cell; and

(d) regenerating a plant from the transformed cell of step (c), to produce the variant

plant.

The above method is also used to produce a plant variety in a non-geranium or non-rose plant species characterized by reduced expression or activity of an ACC synthase enzyme and reduced ethylene production compared to a conventional variety of the species, comprising producing a variant plant as above, and selfing the plant to generate the variety.

This invention also provides a method for genetically altering a plant, preferably (but not necessarily) a plant of a low RNA species, comprising the steps of:

(a) isolating mRNA of the plant using the 2-butoxyethanol precipitation technique wherein at least about 3-5 grams of plant tissue starting material is used to attain a critical mass amount of RNA for precipitation;

(b) constructing a cDNA library from the isolated mRNA

(c) identifying and cloning a desired DNA sequence from the library

(d) genetically altering the cloned DNA sequence;

(e) transforming cells of the plant or the plant directly with the altered DNA sequence; and

(f) if done through a cell-based technique, reproducing a plant from the cells which plant expresses the altered DNA sequence,

thereby genetically altering the plant.

In the above, method the plant is preferably a species of the genus *Pelargonium* or *Rosa*. In the above method, the cloned DNA sequence preferably encodes ACC synthase. The cDNA in the above method is preferably selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3 for geranium, and SEQ ID NO:14 for rose.

The above method is used to produce a genetically altered geranium or rose plant, comprising the steps of:

(a) isolating geranium mRNA using a 2-butoxyethanol precipitation technique

wherein at least about 3-5 grams of plant tissue starting material is used to attain a critical mass amount of RNA for precipitation;

(b) constructing a cDNA library from the isolated mRNA

(c) identifying and cloning at least one DNA sequence from the library

(d) genetically altering the cloned DNA sequence;

(e) transforming geranium or rose cells with the altered DNA sequence; and

(f) regenerating the genetically altered geranium or rose plant from the cells, which plant expresses the altered DNA sequence.

The invention is further directed to a method of isolating plant mRNA, comprising the steps of:

(a) extracting nucleic acids from a sufficient amount of plant tissue starting material to attain a critical mass amount of RNA for precipitation;

(b) isolating RNA from the nucleic acids of step (a) using a 2-butoxyethanol precipitation technique;

(c) contacting the RNA with a binding partner for mRNA, for example oligo-dT or another molecule or entity which has the characteristics of binding specifically to mRNA with the exclusion of other forms of RNA or DNA. The binding partner may be immobilized on a solid phase or carrier; this yields immobilized mRNA; and

(d) eluting the immobilized mRNA from the carrier by conventional elution methods, or obtaining bound mRNA, thereby isolating the mRNA from total RNA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the ethylene biosynthetic pathway including the step catalyzed by ACC synthase.

Figure 2 is a diagram showing the details of steps of cDNA synthesis from

mRNA

Figure 3 shows the nucleotide sequence of the cDNA clone designated pPHSacc41 (SEQ ID NO:1). The following landmarks are indicated: the short 5' sequence originating in the vector are in italics and underscored; the start ATG codon is in bold and underscored; the termination codon of the coding sequence (TAA or TAG) is in bold and double underscored; the polyadenylation signal (sequence) near the 3' end is shown in bold (AATAAA or AAATAA).

Figure 4 shows the nucleotide sequence of the cDNA clone designated pPHSacc44 (SEQ ID NO:2). Landmarks are as shown in figure 3.

Figure 5 shows the nucleotide sequence of the cDNA clone designated pPHSacc49 (SEQ ID NO:3). Landmarks are as shown in figure 3.

Figure 6 shows the deduced amino acid sequence (SEQ ID NO:4) encoded by nucleotide sequence SEQ ID NO:1.

Figure 7 shows the deduced amino acid sequence (SEQ ID NO: 5) encoded by nucleotide sequence SEQ ID NO:2.

Figure 8 shows the deduced amino acid sequence (SEQ ID NO:6) encoded by nucleotide sequence SEQ ID NO:3.

Figure 9 shows the nucleotide sequence of the cDNA clone designated pRoseKacc7 (SEQ ID NO:14). The following landmarks are indicated: the start ATG codon is in bold and underscored; the termination codon of the coding sequence (TAG) is in bold and double underscored.

Figure 10 shows the deduced amino acid sequence (SEQ ID NO:15) encoded by nucleotide sequence SEQ ID NO:14.

BEST MODE FOR CARRYING OUT THE INVENTION

The present inventor has isolated, cloned and identified several cDNA sequences encoding the enzyme ACC synthase in both geranium and rose plants (specifically from

Pelargonium hortorum cv Sincerity and *Rosa*). These cDNA sequences correspond to genes which are important in the control of ethylene production. The DNA is expressed in any of a number of expression systems, including an *in vitro* expression system to yield a polypeptide product which preferably has ACC synthase enzymatic activity.

Cloned ACC synthase gene(s) or fragments thereof, when introduced in reverse orientation (antisense) under control of a strong promoter (discussed below in detail), such as the cauliflower mosaic virus promoter CaMV35S, can be used to genetically modify either a geranium or a rose plant. Selected antisense sequences sharing sufficient homology to ACC synthase genes in other plants can be used to achieve similar genetic modification. One result of this modification is a reduction in the amount of translatable ACC synthase-encoding mRNA. As a consequence, the amount of ACC synthase produced in the plant cells is reduced, thereby reducing the rate of conversion of ACC to ethylene. This genetic modification can effect a permanent change in ethylene levels in the modified plant and be propagated in offspring plants by selfing or other reproductive schemes. Hence, the invention provides a plant modified as described herein as well as plants which, although modified in a different manner achieve similar results or utilize similar concepts as disclosed herein. The genetically altered plant is used to produce a new variety or line of plants wherein the alteration is stably transmitted from generation to generation.

The geranium plant is one of the most ethylene-sensitive flowering plants (Nell, T.A., 1993, In: White, J.W., ed., *Geranium IV. The Growers Manual*, Edition Four, Ball Publishing, Geneva, IL, pp 171-172). A change in ethylene level may thus have a great impact on its commercial desirability. The present invention provides isolated ACC synthase genes obtained specifically from geranium for use in genetic modification preferably of geranium plants and isolated ACC synthase genes obtained specifically from rose for use in genetic modification preferably of rose plants. The full length DNA molecules described herein are unique to geraniums and to roses and vary significantly in sequence from ACC synthase DNA in any other unrelated plant species.

Because of such interspecies variation, to achieve stable genetic modification, it

may be important that an ACC synthase gene or gene fragment (a) be obtained from the same species or (b) be a functional derivative of the DNA sequence native to the species. However, it is possible that a selected sequence from one plant genus or species may be employed using antisense technology in a different genus or species to achieve a useful effect such as that described here. The present invention thus provides for the first time the appropriate DNA sequences which may be used to achieve a stable genetic modification primarily of geranium and rose plants (and of other plants as well).

For the identification, in general, preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, Southern blots, Northern blots after separation of the RNA on a formaldehyde agarose gel, DNA ligation and bacterial transformation were carried out using conventional methods well-known in the art. See, for example, Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989.

As used herein, the term "plant" refers to either a whole plant, a plant part, a plant cell, or a group of plant cells. The types of plants which can be used in the method of the invention generally includes the genus *Pelargonium* (geraniums) and the genus *Rosa* (roses) which can take up and express the DNA molecules of the present invention. It may include plants of a variety of ploidy levels, including haploid, diploid, tetraploid, and polyploid.

A "transgenic plant" is defined as a plant which is genetically modified in some way, including but not limited to a plant which has incorporated heterologous DNA or modified DNA or some portion of heterologous or homologous DNA into its genome. The altered genetic material may encode a protein, comprise a regulatory or control sequence, or may comprise an antisense sequence or encode an antisense RNA which is antisense to an endogenous DNA or mRNA sequence of the plant. A "transgene" or a" transgenic sequence" is defined as a foreign or atypical gene or partial sequence which has been incorporated into a transgenic plant.

As used in the present application, the term "substantial sequence homology" or "substantially homologous" is used to indicate that a nucleotide sequence (in the case of

DNA or RNA) or an amino acid sequence (in the case of a protein or polypeptide) exhibits substantial functional or structural equivalence with another nucleotide or amino acid sequence. Any functional or structural differences between sequences having substantial sequence homology will be *de minimis*; that is, they will not affect the ability of the sequence to function as indicated in the desired application. Differences may also be simply due to inherent variations in codon usage among different species. Sequences that have substantial sequence homology with the sequences disclosed herein are usually "variants" of the disclosed sequence, such as mutations, but may also be synthetic sequences. Structural differences are considered *de minimis* if there is a significant amount of sequence overlap or similarity between two or more different sequences or if the different sequences exhibit similar physical characteristics even if the sequences differ in length or structure. Such characteristics include, for example, ability to hybridize under defined conditions, or, in the case of proteins, immunological crossreactivity, similar enzymatic activity, *etc.*

Additionally, two nucleotide sequences are substantially homologous if the sequences have at least 70 percent, more preferably 80 percent and most preferably 90 percent sequence similarity between them. Two amino acid sequences are substantially homologous if they have at least 50 percent, preferably 70 percent, and most preferably 90 percent similarity between the active portions of the polypeptides.

The term "hybridization" as used herein is generally understood to mean hybridization at appropriate conditions of stringency as would be readily evident to those skilled in the art depending upon the nature of the probe sequence and target sequences. Conditions of hybridization and washing are well-known in the art, and the adjustment of conditions depending upon the desired stringency by varying incubation time and temperature and ionic strength of the solution are readily accomplished. See, for example, Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989). The choice of conditions is dictated by the length of the sequences being hybridized, in particular the length of the probe sequence, the relative G-C content of the nucleic acid and the amount

of mismatches to be permitted. Low stringency conditions are preferred when partial hybridization between strands that have lesser degrees of complementarity is desired. When perfect or near-perfect complementarity is desired, high stringency conditions are preferred. For typical high stringency conditions, the hybridization solution contains 6X SSC, 0.01M EDTA, 5X Denhardt's solution and 0.5% SDS. Hybridization is carried out at about 68°C for 3-4 hours for fragments of cloned DNA and 12-16 hours for total eukaryotic DNA. For lower stringency, the temperature is reduced to about 12°C below the melting temperature (T_m) of the duplex. The T_m is known to be a function of G-C content and duplex length as well as the ionic strength of the solution.

By "functional derivative" of a nucleic acid (or poly- or oligonucleotide) is meant a "fragment," "variant," "homologue" or "analogue" of the gene or DNA sequence encoding ACC synthase, or in some way related to the production or use of ACC synthase, especially geranium or rose ACC synthase. A functional derivative may retain at least a portion of the function of the ACC synthase-encoding DNA which permits its utility in accordance with one embodiment of the present invention. Such function may include the ability to hybridize with native geranium, native rose, or homologous DNA from another plant which encodes ACC synthase or with an mRNA transcript thereof, or, in antisense orientation, to inhibit the transcription and/or translation of geranium ACC synthase mRNA, rose ACC synthase mRNA, or the like.

A "fragment" of the gene or DNA sequence refers to any subset of the molecule, that is, a shorter polynucleotide- or oligonucleotide. A "variant" refers to a molecule substantially similar to either the entire gene or a fragment thereof, such as a nucleotide substitution variant having one or more substituted nucleotides but which maintains the ability to hybridize with the particular gene or to encode a mRNA transcript which hybridizes with the native DNA. A "homologue" refers to a fragment or variant sequence from a different plant genus or species. An "analogue" refers to a non-natural molecule substantially similar to or functioning in relation to either the entire molecule, the variant, or to a fragment thereof.

"Altered" expression" or an "alteration" of expression of a gene (most particularly

of ACC synthasc), as used herein, refers to any process or result whereby the normal expression of the genc, for example that occurring in an "unmodified" geranium or rose plant, defined as a known, conventional, naturally-occurring geranium or rose plant, is changed in some fashion. As intended herein, an alteration is a complete or preferably a partial reduction in the expression of ACC synthase, but may also include a change in the timing of expression, or another state wherein the expression of ACC synthase differs from that which would be most likely to occur naturally in an unmodified geranium or rose plant, variety or cultivar. A preferred alteration is one which results in a decrease in ethylene production by the plant compared to ethylene production in an unmodified plant.

In producing a genetically altered plant according to this invention, it is preferred to select individual plantlcts or plants by the desired trait, generally reduced ACC synthesis expression and reduced ethylene production. Expression of ACC synthase can be measured by quantitating the amount of ACC synthase mRNA using conventional hybridization techniques. Alternatively, the amount of ACC synthase protein can be quantitated, for example in a conventional immunoassay method using a specific antibody such as those described herein. Finally, the ACC synthase enzymatic activity can be measured using biochemical methods as described in Kionka *et al.*, *supra*; Amrhein *et al.*, *supra*; or Hoffman N.E., *et al.*, *supra*. Ethylene biosynthesis in thc plantlet or plant can be quantitated using known methods Yang, S.F. *et al.* (1984), *Annu. Rev Plant Physiol*:35, 155-189); Abeles, F.B. *et al.* eds, *Ethylene in Plant Biology*, Academic Press, New York, 1976White, J.W., ed., *Geranium IV. The Growers Manual*, Edition Four, Ball Publishing, Geneva, IL.

In order for a newly inserted gene or DNA sequence to be expressed, resulting in production of the protein which it encodes (or, in the case of antisense DNA, to be transcribed, resulting in an antisense RNA molecule), the proper regulatory signals should be present in the proper location with respect to the coding or antiscnse sequence. These regulatory signals may include a promotcr region, a 5' non-translated leader sequence and a 3' polyadenylation sequence as well as enhancers and other known

regulatory sequence. The promoter is a DNA sequence that directs the cellular machinery to transcribe the DNA to produce RNA. The promoter region influences the rate at which the mRNA product and, if the DNA encodes a protein, the resultant protein product, are made. The 3'-polyadenylation signal is a non-translated region that functions in plant cells to cause the addition of a polyadenylate stretch to the 3' end of the mRNA to stabilize it in the cytoplasm for subsequent translation.

A promoter DNA sequence is operably linked to a second DNA sequence and regulates its transcription. If the second DNA sequence encodes a protein, the promoter DNA sequence is said to be "operably linked" if it affects the transcription of the mRNA encoding the protein product from the second DNA sequence. A DNA sequence comprising a promoter is generally physically near the coding sequence in the same recombinant construct, though physical contiguity is not required. "Strong" promoters are able to direct RNA synthesis at higher rates than weaker promoters. Certain promoters direct RNA production at higher levels only in particular types of cells and tissues. Promoters that direct RNA production in many or all tissues of a plant without the need for "induction" by a specific inducer substance are called constitutive promoters. The operation of a constitutive promoter is relatively independent of the developmental stage of the cell in which it is contained and is most preferred for the present invention. An inducible promoter is one which, in response to the presence of an inducer, is activated. Hence, a coding sequence driven by an inducible promoter can be turned on or off by providing or withdrawing the inducer. A promoter may be homologous, derived from the same species as the coding sequence. Preferably, the promoter is heterologous, that is, derived from another species, or even from a virus.

Expression levels from a promoter which is useful for the present invention can be tested using conventional expression systems, for example, by measuring levels of a reporter gene product (protein or mRNA) in extracts of the leaves, stems, roots and flowers of a transgenic plant into which the promoter/reporter have been introduced.

Cauliflower mosaic virus (CaMV) is a double-stranded DNA plant virus. It contains two promoters responsible for the production of transcripts of 35S and 19S in

size in infected plants (Guilley, H., *et al.*, *Cell 30*:763 (1982)). The 35S promoter (CaMV35S) is one of the strongest constitutive heterologous promoters known in plants (Odell, *et al.*, *Nature 313*:810-812 (1985); Jensen, *et al.*, *Nature 321*:669-674 (1986); Jefferson, *et al.*, *EMBO J. 6*:3901-3907 (1987); Kay, *et al.*, *Science 236*:1299-1302 (1987); Sanders, *et al.*, *Nucl. Acids Res. 4*:1543-1558 (1987)). Two different domains within the CaMV 35S promoter may differentially regulate expression of a coding sequence in different plant tissues (domain A, from nucleotides -90 to +8) vs. domain B from nucleotides - 343 to -90) , as described by Benfey, *et al.*, 1989 *EMBO J. 8*:2195-2202.)The CaMV35S promoter is active in isolated protoplasts (Fromm, M., *et al.*, *Proc. Natl. Acad. Sci. USA* 82:5824 (1985)) and is expressed in all organs of various transgenic plants in the absence of any viral protein, making it widely used in plant genctic engineering.

Because of variability in the expression of genes driven by the CaMV35S promoter, (which may be either an intrinsic property of the promoter or a result of variability in the position at which CaMV35S promoter-driven DNA sequence is integrated into the genome of the transformed plant), CaMV35S may be particularly useful for effecting different degrees of altered gene expression by an antisense sequence which the promoter controls. Additional useful plant promoters in, for example, other caulimoviruses (a group of double-stranded DNA viruses to which the cauliflower mosaic virus belongs) have also been developed and are useful for similar applications. Two caulimoviruses distantly related to CaMV are the figwort mosaic virus (FMV) (Richins, *et al.*, *Nucl. Acids Res. 15*:8451-8466 (1987)) and the carnation etched ring virus (CERV) (Hull, *et al.*, *EMBO J. 5*:3083-3090 (1986). The promoters of FMV and CERV which are homologues of the CaMV35S promoter are described in Rogers, U.S. Patent No. 5,378,619. Any of the foregoing viral promoters, as well as other viral promoters which act as strong promoters for expression of plant DNA sequences in plant cells, may be used to drive the expression of the DNA molecules of the present invention.

Certain other strong plant promoters are also useful to direct the expression of the ACC synthase DNA (or antisense sequences) of the present invention. For example, the

small subunit (SSU) of the enzyme ribulose-1,5-bisphosphate carboxylase (RuBPCase), the primary enzyme of the carbon fixation pathway in chloroplasts of plants of the C3 class is an example of a polypeptides known to be highly expressed in plants. A highly efficient SSU promoter DNA such as the promoter DNA from the SSU gene denominated SSU301 from Petunia (Bedbrook, *et al.*, U.S. Patent No. 4,962,028) may be used herein. The promoter may be used in the form of an isolated 5' fragment of the SSU gene, and preferably has the 3' end of the fragment modified to create a restriction site which permits ready fusions with the ACC synthase antisense DNA of the present invention. The promoter may be conveniently arranged to form an expression cassette comprising a 5' fragment (the promoter region of the SSU gene), a 3' fragment and a linker region connecting the two fragments. The fusion points between the 5' fragment and the linker region and between the 3' fragment and the linker region are preferably modified to create restriction sites which permit the antisense DNA of the present invention to be substituted for the linker so as to yield "chimeric" genes containing the complete proximal 5' and 3' regions of the SSU gene but none of the SSU coding sequence

Other plant promoter enhancer/sequences which may be used in accordance with the present invention have been described in the following references: Coruzzi, *et al.*, 1984, *EMBO J. 3*:1671-1680; Herrera-Estrella, *et al.*, 1984, *Nature 310*:115-120; Apel, *et al.*, 1978, *Eur. J. Became. 85*:581-588; Stiekema, *et al.*, 1983, *Plant Physiol. 72*:717-724; Thompson, *et al.*, 1983, *Planta 158*:487-500; Jones, *et al.*, 1985, *EMBO J. 4*:2411-2418; Stockhaus, *et al.*, 1989, *Plant Cell 1*:805-814; Gurley, *et al.*, 1986, *Mol. Cell Biol. 6*:559-565; Landsmann, *et al.*, 1988, *Mol. Gen. Genet. 214*:68-73; Bevan, *et al.*, 1989, *EMBO J. 8*:1899-1906; Benfey, *et al.*, 1989, *Science 244*:174-181.

Additionally, certain bacterial promoters have been observed to be expressed in plants, including the *Rhizobium meliloti* FIXD gene promoter (Puhler, *et al.*, U.S. Patent No. 4,782,022) and the nopaline synthase promoter (Ha, *et al.*, 1989, *Nucl. Acids Res. 17*:215-224; An *et al.*, 1988, *Plant Physiol. 88*:547-552). Several promoter sequences, termed the *rol A, B* and *C* promoters, have been identified in *Agrobacterium rhizogenes*

(Schmulling, *et al.*, 1989, *Plant Cell 1*:665-670; Sugaya, *et al.*, 1989, *Plant Cell Physiol. 30*:649-654).

To test the activity of a promoter, E. coli β-glucuronidase (GUS) coding sequence or a mutant Arabidopsis EPSP synthase gene which encodes an enzyme tolerant of glyphosate herbicides may be used as a reporter gene. Transformed plant cells or plants containing the GUS gene operably linked to the promoter being tested are assayed using a histological staining procedure to determine GUS activity in the transformed cells.

The present invention provides antisense oligonucleotides and polynucleotides complementary to the gene or genes encoding ACC synthase in geranium or rose plants. Such antisense oligonucleotides, should be at least about six, ten, twenty, or fifty nucleotides in length to provide minimal specificity of hybridization, and may be complementary to one strand of DNA or to mRNA encoding ACC synthase (or to a portion thereof), or to flanking sequences in genomic DNA which are involved in regulating ACC synthase gene expression. The antisense oligonucleotide may be as large as about 100 nucleotides, an may extend in length up to and beyond the full coding sequence for which it is antisense. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded.

The action of the antisense nucleotide may result in specific alteration, primarily inhibition, of ACC synthase gene expression in cells. For a general discussion of antisense, see: Alberts, B., *et al.*, *MOLECULAR BIOLOGY OF THE CELL*, 2nd Ed., Garland Publishing, Inc., New York, NY (1989), in particular, pages 195-196, which reference is hereby incorporated by reference.

The antisense oligonucleotide may be complementary to any portion of the ACC synthase encoding sequence. In one embodiment, the antisense oligonucleotide may be between about 6, 10, 20, or 50 and 100 nucleotides, and may be complementary to the initiation ATG codon and an upstream, non-coding translation initiation site of the ACC synthase sequence. For example, antisense nucleotides complementary primarily for non-coding sequence, are known to be effective inhibitors of the expression of genes

encoding transcription factors (Branch, M.A., 1993 *Molec. Cell. Biol. 13*:4284-4290).

Preferred antisense oligonucleotides are complementary to a portion of the mRNA encoding ACC synthase. For instance, it is expected that by introducing a full length cDNA clone gene in an antisense orientation, successful alteration of gene expression will be most probable. Naturally, introduction of partial sequences, targeting to specific regions of the gene, and the like can be effective as well. An example of a preferred antisense oligonucleotide for geranium is a 50mer which is antisense to 50 nucleotides in the 5' half of an RNA transcript of an ACC-encoding cDNA (such as SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3), more preferably any stretch of 50 nucleotides in the first 500 nucleotides of the 5' part of the RNA transcript. An example of a preferred antisense oligonucleotide for rose is similarly a 50mer which is antisense to 50 nucleotides in the 5' half of an RNA transcript of an ACC-encoding cDNA (such as SEQ ID NO:14), more preferably any stretch of 50 nucleotides in the first 500 nucleotides of the 5' part of the RNA transcript. For example, the antisense oligonucleotide can be antisense to nucleotides 1-50, 2-51, 3-52, 4-53,5-54, *etc.*, of the RNA transcript. Alternatively, the antisense oligonucleotide can be shorter for wither plant, for example a 30-mer, and be antisense to any 30 nucleotide stretch of the RNA transcript, preferably in the first 500 5' nucleotides.

As is readily discernible by one of ordinary skill in the art, the minimal amount of homology required by the present invention is that sufficient to result in sufficient complementarity to provide recognition of the specific target RNA and inhibition or reduction of its translation or function while not affecting function of other mRNA molecules and the expression of other genes. While the antisense oligonucleotides of the invention comprise sequences complementary to at least a portion of an RNA transcript of ACC synthase, absolute complementarity, although preferred, may not be required. A sequence "complementary to at least a portion of" another sequence, as referred to herein, may have sufficient complementarity to be able to hybridize with that of other sequences *in vivo*, perhaps forming a stable duplex. Naturally, the ability to hybridize may depend on both the degree of complementarity and the length of the antisense

nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with the ACC synthase target sequence it may contain and still form a stable duplex. One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting temperature of the hybridized complex as discussed above and other techniques.

The antisense RNA oligonucleotides may be generated intracellularly by transcription from exogenously introduced nucleic acid sequences. Thus, antisense RNA may be delivered to a cell by transformation or transfection or infection with a vector, such as a plasmid or a virus, into which is incorporated (a) DNA encoding the antisense RNA and operably linked thereto (b) the appropriate regulatory sequences, including a promoter, to express the antisense RNA in a target host cell (and whole plant). Within the cell the exogenous DNA or a portion thereof may be transcribed, producing an antisense RNA of the invention. Vectors can be plasmid, viral, or others known in the art which are used for replication and expression in plant cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in plant, preferably geranium or rose, cells. Such promoters can be inducible or preferably are constitutive as described above. Such a vector, preferably a plasmid, becomes chromosomally integrated such that it can be transcribed to produce the desired antisense RNA. Such plasmid or viral vectors can be constructed by recombinant DNA technology methods that are standard in the art.

An oligonucleotide, between about 6 and about 100 bases in length and complementary to the target sequence of ACC synthase, as described above may be prepared by chemical synthesis from mononucleotides or shorter oligonucleotides, or produced by recombinant means.

Basic procedures for constructing recombinant DNA and RNA molecules in accordance with the present invention are disclosed by Sambrook, J., *et al.*, In: *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), which reference is herein incorporated by reference. Oligonucleotide molecules having a strand which encodes antisense RNA complementary to an ACC

synthase sequence can be prepared using procedures which are well known to those of ordinary skill in the art. Details regarding such procedures are described in: Belagaje, R., *et al., J. Biol. Chem. 254*:5765-5780 (1979); Maniatis, T., *et al.*, In: *MOLECULAR MECHANISMS IN THE CONTROL OF GENE EXPRESSION*, Nierlich, D.P., *et al.*, eds., Acad. Press, N.Y. (1976); Wu, R., *et al., Prog. Nucl. Acid Res. Molec. Biol. 21*:101-141 (1978); Khorana, H.G., *Science 203*:614-625 (1979)). Automated synthesizers may be used for DNA synthesis (such as are commercially available from Biosearch, Applied Biosystems, *etc.*).

Techniques of nucleic acid hybridization are disclosed by Sambrook *et al.* (*supra*), and by Haymes, B.D., *et al.*, In: *NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH*, IRL Press, Washington, DC (1985)), which references are herein incorporated by reference.

The transgenic plants of the present invention may be prepared by DNA transformation using any method of transformation known in the art. These methods include transformation by direct infection or co-cultivation of plants, plant tissue or cells with *Agrobacterium tumefaciens* (Horsch, *et al., Science 225*:1229 (1985); Marton, *Cell Culture and Somatic Cell Genetic of Plants 1*:514-521 (1984)); Fry, *et al., Plant Cell Reports 6*:321-325 (1987); direct gene transfer into protoplasts or protoplast uptake (Paszkowski, *et al., EMBO J. 12*:2717 (1984); Loerz, *et al., Mol. & Gen. Genet. 178*:1199 (1985); electroporation Fromm, *et al., Nature 319*:719 (1986)); microprojectile or particle bombardment (Klein, *et al., Bio/Technology 6*:559-563 (1988)); injection into protoplasts cultured cells and tissues (Reich *et al., Bio/Technology, 4*:1001-1004 (1986)); or injection into meristematic tissues of seedlings and plants (De La Pena, *et al.*, Nature, 325:274-276 (1987); Graves, *et al., Plant Mol. Biol. 7*:43-50 (1986); Hooykaas-Van Slogteren, *et al., Nature 311*:763-764 (1984); Grimsley, *et al., Bio/Technology 6*:185 (1988); and Grimsley, *et al., Nature 325*:177 (1988).

The *Agrobacterium tumefaciens* strain 208 carrying the disarmed pMP90RK plasmid can be used to achieve transformation. Used for plant transformations, the vector plasmid may be introduced into the Agrobacterium by the triparental conjugation system

(Ditta, *et al.*, (1980) *Proc. Natl. Acad. Sci. USA* 77:7347-7451) using the helper plasmid pRK2013. The vectors may be transferred to plant cells by the *vir* functions encoded by the disarmed pMP90RK Ti plasmid. The vector is opened at the pTiT37 right border sequence and the entire vector sequence is inserted into the host plant chromosome. The pMP90RK Ti plasmid is probably not transferred to the plant cell but remains in the *Agrobacterium*.

Normally, regeneration will be involved in obtaining a whole plant from the transformation process. The term "regeneration" as used herein, means growing a whole plant from a plant cell, a group of plant cells, a plant part or a plant piece (e.g. from a protoplast, callus, tissue part, or explant, etc.) Plant regeneration from cultured protoplasts is described in Evans, *et al.*, *Handbook of Plant Cell Cultures 1*:124-176 (MacMillan Publishing Co. New York 1983); Davey, M.R., *Protoplasts* (1983), Lecture Proceedings, pp.12-29, Birkhauser, Basel, 1983); P.J. Dale, *ibid*, at pp. 31-41, (Birkhauser, Basel 1983); and H. Binding, *Plant Protoplasts*, pp.21-73, CRC Press, Boca Raton, 1985).

Plant parts obtained from the regenerated plant in which expression of an ACC synthase gene has been altered, such as flowers, seeds, leaves, branches, fruit, and the like are included within the definition of "plant" as stated above, and are included within the scope of the invention. Progeny and variants and mutants of the regenerated plants are also included, especially if these parts comprise the introduced DNA sequences.

The present invention also provides ACC synthase proteins encoded for by the cDNA molecules described above. For geranium, such proteins preferably have the amino acid sequences SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6 as shown in figures 6, 7, and 8. For rose, such proteins preferably have the amino acid sequence of SEQ ID NO:15 as shown in figure 10. In each case, these proteins, or functional derivatives thereof, are preferably produced by recombinant methods optionally in combination with chemical methods.

A "functional derivative" of the ACC synthase protein is a "fragment," "variant," "analog," or "chemical derivative" of ACC synthase, which retains at least a portion of

the function of the ACC synthase which permits its utility in accordance with the present invention. Such function includes enzymatic activity or immunological crossreactivity with an antibody specific for ACC synthase. A fragment of the ACC synthase protein refers to any subset of the molecule, that is, a shorter peptide. A variant refers to a molecule substantially similar to either the entire protein or a fragment thereof. Variant peptides may be conveniently prepared by direct chemical synthesis using methods well-known in the art. An "analog" of ACC synthase refers to a non-natural protein substantially similar to either the entire protein or a fragment thereof. A chemical derivative of ACC synthase contains additional chemical moieties not normally a part of the protein or peptide fragment thereof. Covalent modifications of an ACC synthase peptide are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

A protein or peptide according to the present invention may be produced by culturing a cell transformed with a DNA sequence of this invention, allowing the cell to synthesize the protein, and obtaining the protein from the culture medium if it is secreted, or if it is intracellular, obtaining it by extraction. In a preferred embodiment, the protein is produced in a cell free system, for example, as described by Ranu, R.S., *et al,* 1979, *Meth. Enzymol. 60*:459-484 and Ranu, R.S., *et al,* (1996) *Gene Expression 5*:143-153.

To produce an isolated, purified protein or peptide, the *in vitro* translation product or the cell or tissue extract from transformed plant cells or plant parts is subjected to conventional biochemical purification methods, including but not limited to affinity chromatography using an antibody specific for an epitope of the protein.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLES

Plant Material

Pelargonium hortorum cv sincerity (geranium) plants and *Rosa* (rose) plants grown and maintained in a greenhouse were used to clone the cDNA corresponding to ACC synthase genes. Flower tissue in the form of senescing flower petals (from differentstages) were collected in liquid nitrogen and used immediately or stored at -70°C until

Messenger RNA (mRNA) isolation

The quality of the mRNA largely determines the quality of cDNA library generated subsequently for cDNA cloning of ACC synthase genes. By "quality of the mRNA" is intended the presence of all the desired mRNA species, especially those mRNA molecules that are present in cells in relatively low abundance (either because of the number of gene copies, the rate of transcription or the stability of the mRNA). The most widely used method for preparation of RNA utilizes extraction with 4 M guanidine thiocyanate of total RNA (Chomczynski, P., *et al.* (1987), *Anal. Biochem.*. 162:156-159). When this method was tried by the present inventor for geranium, the quality of RNA obtained was inadequate and did not permit a generation of a useable, high quality cDNA library (containing cDNA inserts corresponding to the least abundant mRNAs). Thus, when cDNA libraries prepared using the conventional method were screened for the presence of cDNA inserts encoding ACC synthase, the clones identified contained only partial genes or, mostly frequently, false positives. This problem alone made the process of isolating the ACC synthase genes of this invention extremely difficult and challenging.. This conclusion was also suggested from the results of expression screening of such libraries with antibodies specific for the ACC synthase protein. In sum, the prior art RNA isolation technique at best invited experiments to try to find the full length genes, but provided no reasonable expectation of success. Problems posed by the poor quality of the total RNA prepared using conventional methods led the present inventor to look for alternative means for obtaining RNA of sufficiently high quality to be useful for the purposes of this invention, namely preparation of a cDNA library having a high probability of including a full length DNA sequences corresponding to low-abundance mRNAs, in particular full-length ACC synthase coding sequences.

Preparation of RNA

The preferred method discovered by the present inventor was based on the precipitation of RNA from a tissue extract using 2-butoxyethanol (Manning, K., 1991, *Anal. Biochem. 195*:45-50) with modifications. This method is referred to herein as "a 2-butoxyethanol precipitation technique." This technique was originally developed for RNA isolation, and by adapting it for mRNA isolation, the extraordinary results of this invention were achieved. Generally, in order to achieve the required RNA precipitation, a co-precipitation critical mass of RNA must be present in the preparation. The relative low proportion of RNA in relation to the total extracted material required the recognition by the present inventor that the standard amount of tissue extract used in RNA preparation, about 1 gram or less, would be insufficient for certain types of plants such as geranium and rose (discussed more fully below). The success described herein was ultimately attained by using an unusually large amount of tissue. For effort with geranium, this was about 3-5 grams. For effort with rose, this was also about 3-5 grams. While, in hindsight, this may seem like a simple problem and solution, in fact, this problem does not appear to have been considered by others, and, therefore, the novel method is not an obvious modification of the older technique.

This problem in part stems from the fact that the desired precipitation is "non-linear," meaning that no simple linear relationship exists between the mass of RNA and the amount of precipitation. Rather, the process is a threshold phenomenon, and unless that critical mass is present, precipitation will not occur. For these reasons, the prior art technique would appear on its face to be inapplicable for obtaining a high quality mRNA preparation from woody plants such as geranium or rose. Surpassing such a critical amount of RNA, that is, an amount at which precipitation occurs, permitted the method, as modified, to demonstrate its full utility. Hence, the present inventor achieved an unexpected and extraordinary result, in spite of the fact that the technology underlying the modifications introduced to earlier methods had been available. Those of ordinary skill in the art may have appreciated (although this is not evident) that a key impediment was in the obtaining of high quality mRNA to generate a fully representative cDNA

library. Furthermore, a long felt need in the art for such a library had not been satisfied. Nevertheless, substantial attempts in the prior art failed because practitioners did not understand the true nature of the reasons for failure of this type of technique.

The present inventor's discovery of a means to here achieve the co-precipitation critical mass of RNA is particularly important to the class of plants which have a low proportion of RNA in their tissue, such as less than only 1/10,000th of the total tissue usually obtained. It is also particularly important for woody plants such as geranium or rose, for which the present invention is particularly useful. These groups of plants comprises plant species that have a low proportion of RNA in their tissue relative to non-nucleic acid material. This is in contrast to other plants which have a higher proportion of RNA and are amenable to the preparation of high quality mRNA (and cDNA corresponding thereto) by the traditional approaches of the prior art. While this "low RNA" group of plants is known to include at least *Pelargonium* species and *Rosa* (rose) species, it is clear that other plants also fall in this category, as would be evident to those skilled in the art. This group of plants is characterized in one manner as being woody (that is, they contain large amounts of fiberous material) and therefore having a low relative abundance of RNA, or conversely, as a high relative proportion of non-nucleic acid material. Thus, in this category of low RNA plants, it would be necessary to use a "large" amount of tissue, namely, an amount which (depending upon the particular plant or technique) is sufficient to yield a co-precipitant critical mass of total RNA in the process. For *Pelargonium, Rosa,* and the like, a co-precipitant critical mass of RNA is about 200 μg for successful implementation of the 2-butoxyethanol precipitation technique described herein. (Other RNA isolation techniques or plants may, of course, each have their own critical mass, that is, the presence of enough total RNA for precipitation to actually occur.) Thus, for the present technique and plants, about 3-5 grams of flower tissue was used initially. This may represent a minimum amount for some plants. Naturally more would also work.

The flower tissue was ground into a powder using a pestle and mortar precooled by liquid nitrogen. The resulting material was then ground with 12-20 ml of extraction

buffer (0.2M boric acid/Tris-HCl and 10 mM EDTA (pH 7.6)), followed by addition of 0.24-0.4 ml of 25% sodium dodecyl sulfate (SDS) and 0.24-0.4 ml of 2-mercaptoethanol (2-ME).

The mixture was brought to room temperature and extracted with an equal volume of extraction buffer, saturated phenol/chloroform mixture. The mixture was centrifuged at 20,000xg at room temperature. The upper aqueous phase was collected and kept in a fresh tube. The interphase and lower organic phase were re-extracted with an equal volume of extraction buffer containing SDS and 2-ME. After centrifugation at 20,000xg, the second aqueous phase was removed and combined with the first aqueous phase. The pooled aqueous phase was diluted with 2.5 volume of water and a quantity of 1M sodium acetate (pH 4.5) sufficient to make the final concentration 80 mM.

This was followed by addition of 0.4 volumes of 2-butoxyethanol (2-BE). After 30 minutes on ice, the mixture was centrifuged at 20,000 x g for 10 minutes at 0°C. The clear supernatant was collected. Additional 2-BE was added to bring the total to one volume. After 30 minutes on ice, the nucleic acid-containing pellet was collected by centrifugation at 20,000 x g for 10 minutes at 0°C. The pellet was washed first with a 1:1 (v/v) mixture of extraction buffer and 2-BE, followed by 70% ethanol containing 0.1M potassium acetate (pH 6.0), and finally with 100% ethanol. The pellet was then air dried.

The nucleic acid pellet was dissolved in water to a concentration of about 1mg/ml and sufficient 12M LiCl was added to bring the LiCl concentration to 3M. After one hour on ice, an RNA precipitate was collected by centrifugation at 12,000 x g for 10 minutes at 0°C. The pellet was washed twice with 3M LiCl and once with 70% ethanol and was finally air dried. RNA was dissolved 0.2-0.5 ml of 10mM Tris-HCl, 1mM EDTA (pH 8.0) (TE buffer).

Isolation of mRNA

PolyA$^+$mRNA was isolated by binding to Dynabeads-oligo(dT)25 (Dynal, Inc., Lake Success, NY). The oligo (dT)25 is a preferred binding partner, in addition others are known in the art, the key function being merely the ability to selectively attach to the

mRNA. For this binding partner, the protocol provided by the manufacturer was used. PolyA$^+$RNA was bound to Dynabeads in the presence of 1x binding buffer for 30 minutes. The Dynabeads serve as one of the many possible solid phase supports or carriers. This served to immobilize the mRNA. The beads were washed three times with washing buffer containing lithium dodecyl sulfate (LiDS) and once with wash buffer alone. mRNA was eluted from the beads with 50 µl of TE buffer.

The composition of the buffers was as follows:

(a) 1x Binding Buffer: 10mM Tris-HCl (pH 7.5), 0.5M LiCl, 1mM EDTA, 0.5% LiDS;

(b) Washing Buffer with LiDS: 10mM Tris-HCl, 0.15M LiCl, 1mM EDTA, 0.1% LiDS

Synthesis of cDNA

The mRNA preparation (5µg) isolated as above was used to synthesize cDNA using the ZAP Express® cDNA synthesis system from Stratagene (La Jolla, CA). The details of the steps of synthesis are presented in Figure 2. The first strand synthesis was carried out with murine-Moloney leukemia virus reverse transcriptase (M-MuLV-RT) in the presence of mRNA, a primer containing a 50 base long oligonucleotide

5'-GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTT-3'

*Xho*I [SEQ ID NO:7]

with an *XhoI* restriction recognition site (shown underscored). This allows the finished cDNA to be inserted into the ZAP Express® Vector in the sense orientation (*Eco*RI-*Xho*I) with respect to the LacZ promoter. The poly(dT) region binds to the poly(A) tail of mRNA template and the reverse transcriptase starts the synthesis of first strand. The nucleotide mixture for the synthesis of first strand contained dATP, dGTP, dTTP, and 5-methyldCTP. The first strand has methyl groups on each cytosine base which protects cDNA from restriction enzymes used in subsequent cloning steps.

RNase H nicks the RNA bound to the first strand cDNA to produce multiple fragments which serve as primers for DNA polymerase I (*PolI*). *PolI* nick-translates the RNA fragments into second strand of cDNA. The cDNA ends are blunted in the presence of Klenow fragment and dNTPs. The *EcoRI* adaptors as shown below

5'AATTCGGCAGAG-3' [SEQ ID NO:8]
GCCGTCTCp5'

are ligated to the blunt ends. The *Xho*I digestion of cDNA releases the *Eco*RI adaptor and residual linker-primer from 3'-end of the cDNA. The cDNA is size fractionated on Sephacryl-S400® and then ligated to the ZAP Express Vector® arms.

Only cDNA of 1.5 kb pairs was used to ligate into ZAP Express Vector® and then packaged into bacteriophages using Gigapack® III Gold Packaging extract protocol from Stratagene. The unamplified cDNA library generated in this way was used for subsequent screening for ACC synthase genes.

Development of a Polymerase Chain Reaction (PCR) Probe for the Screening of ACC Synthase Genes.

The first strand of cDNA synthesis was carried out with 2μg of mRNA using the ready-to-go T-Primed First-Strand synthesis protocol obtained from Pharmacia Biotechnology (Piscataway, NJ). The first strand cDNA product was then used to develop a PCR probe. PCR amplification (Mullis, K.B., *et al,* F.A. (1987), *Meth. Enzymol. 155*:355-350) was performed in a Techne PHC-2 Thermocycler (Techne, Princeton, NJ).

The following PCR primers were used for both the Geranium and Rose efforts:

Primer I:

5'-GGIC/TTICCIGGITTC/TC/AGIG/ATIGG-3'

This is alternately designated as:

5'GGNYTNCCNGGNTTYMGNRTNGG3' (where N=inosine) [SEQ ID NO:9]

Primer II:

5'-CAIAIICG/TG/AAAG/CC/AAICCIG/AG/CC/TTC-3'),

This is alternately designated as:

5'CANANNCKRAASMANCCNRSYTC3' (where N=inosine) [SEQ ID NO:10]

The PCR reaction (50µl) contained 5mM Tris-HCl (pH 8.3); 3mM $MgCl_2$, 50mM KCl, 50 pmol of primer I: 3µl of synthesized first strand cDNA, 200mM each of the four dNTPs and 25 units of - (DELTA) *Taq* DNA polymerase (Amersham Life Sciences, Inc., Arlington Heights, IL). Reaction samples were overlaid with 20µl of mineral oil. After an initial denaturation at 95°C for 4 minutes, samples were subjected to two cycles in which conditions were 94°C for one minute for denaturation, 60°C for two minutes for annealing, and 72°C for one minute for extension. It was followed by 30 cycles at 94°C for 30 seconds, 60°C for one minute; and 72°C for 45 seconds. The last cycle was at 72°C for 5 minutes.

On analysis by agarose gel electrophoresis, the amplified DNA showed a DNA band of about 360 bp. The band was localized in the gel under a UV lamp and excised. DNA from the gel was purified by using Spin-Bind Recovery system from FMC BioProducts (Rockland, ME). The DNA was then cloned using the protocol provided by manufacturer into a TA Cloning Vector called pCRII (Invitrogen, San Diego, CA) and then sequenced.

The 360bp fragment cloned in the pCRII vector was excised and used to prepare a [^{32}P]-labeled probe. The Maga Prime system from Amersham Life Science, Inc. (Arlington Heights, IL) was used according to the manufacturer's protocol. The labeled DNA probe incorporated nearly 70% of the input [α^{32}P]dATP.

Isolation of cDNA clones from the cDNA library

Unamplified recombinant bacteriophages (1 x 10^6 pfu) were screened with the [^{32}P] labeled probe. Phages (50,000 pfu) were grown on a 150-mm NZY plate for six hours at 37°C. The plates were cooled to 4°C. Phages were transferred onto a Hybond-N+ nylon membrane (Amersham, Inc.) for 40 seconds. The DNA on membrane was denatured by treatment with 1.5 M NaCl-0.5M NaOH for 2 minutes, neutralized in 1.5M

NaCl-0.5M Tris-HCl (pH 8.0) for 5 minutes and finally washed in 0.2 M Tris-HCl (pH 7.5), 2 x SSC for 30 seconds. DNA was fixed onto the membrane by UV cross-linking (Strategene UV Cross-Linker) and then baked at 80°C for one hour.

The membrane was treated with Rapid-hyb® buffer (Amersham, Inc.) at 55°C for one hour for prehybridization and then probed with [^{32}P]-labeled PCR probe for 3 hours at 55°C. The membranes were washed with 2 x SSC-0.1% SDS for one hour at room temperature and with 0.2-x SSC-0.1% SDS at room temperature. The filters were then exposed to X ray film (Fuji).

For geranium, a total of 95 putative clones were identified during the first screening of the cDNA library. Of these putative clones, 24 were further screened in the second screening cycle at lower density (1000-4000 pfu). Nine putative clones from the second screening were subjected to a tertiary screening. All these nine clones showed strong signal and were judged to be positive. For rose, a total of 33 putative clones were identified during the first screening of the cDNA library. Of these putative clones, eight were further screened in the second screening cycle at lower density (1000-4000 pfu). Eight putative clones from the second screening were subjected to a tertiary screening. All these eight clones showed strong signal and were judged to be positive.

These clones were *in vivo* excised out of the pBK-CMV phagemid vector, and the size of the cDNA insert (representing ACC synthase genes) was determined by electrophoresis. Clones were judged to be nearly full-length, as confirmed by subsequent DNA sequencing.

DNA Sequencing of Clones

The dideoxy chain termination method (Sanger, F., *et al.*, (1977), *Proc. Natl. Acad. Sci. USA 74*:5463-5467)) was used to sequence the ACC synthase cDNA clones for both geranium and rose. This method employed the DELTA *Taq* DNA polymerase protocol developed in the present inventor's laboratory (Ranu, R.S., (1995), *Biotechniques 18*:390-395) or Thermo Sequenase® (Amersham, Inc.). Based on the analysis of the DNA sequence results, the ACC synthase cDNA clones were classified into three classes belonging to two groups for geranium:

Group I

Class 1: pPHSacc41 [SEQ ID NO:1]was 1945 bp in length with an open reading frame (ORF) of 1446 encoding a polypeptide of 52.2 kDa for geranium;

Class 2: pPHSacc44 [SEQ ID NO:2] was 2678 bp in length with an ORF of 1446 bp encoding a 54.2 kDa for geranium;

Group II

Class 3: pPHSacc49 [SEQ ID NO:3] was 1893 bp in length with an ORF of 1470 bp encoding a 55.1 kDa polypeptide for geranium;

All three of the above clones were full-length. Based sequence homologies of the ORF, class 1 and 2 were grouped as Group I and class 3 as Group II. For geranium, groups I and II had 58% nucleotide sequence similarity and the deduced amino acid sequence [SEQ ID NO: 4, 5 and 6, respectively] showed 67% similarity. The DNA sequences of these clones are shown in Figures 3-5. These figures also show various landmarks, including start codon, termination signal and polyA- signal. The deduced amino acid sequences are shown in Figures 6-8.

For rose there was no need for grouping as only one gene appears present. The figure does, however, show the various landmarks, including start codon and termination signal. The deduced amino acid sequence is shown in Figures 15.

Clone pPHSacc44 was unusual in several respects. First, it is 780 bp longer than clone 41. Second it has two distinct poly A signal sequences and polyA "tails" separated by 780 bases of 3' regulatory sequence which are present in genomic DNA. Thus, clone pPHSacc44 appears to include two separate regulatory regions 3' from coding sequence.

Several additional features of these clones and several related clones which include some noteworthy areas as described below. Group I clones included a stop codon (TAG) just before the first AUG codon; the group II clone had a stop codon (TGA) 21

bp upstream (for geranium) of the first AUG. Clone pPHSacc44 had an "extra" 780 bp after a first short (22 bp) 3'-poly(A) tail. Both the poly(A) signal and poly(A) tail were present twice, at the normal 3' untranslated region (3'UTR) and in the extra 3'UTR for it. For rose, the clone included two stop codons on the 5' end of the untranslated region at positions 169 and 178. The regular start codon is at position 271 and the regular stop codon is at position 1711.

Development of Antibody Probes

Antibody probes were prepared for screening a cDNA expression library and for subsequent detection of ACC synthase gene products from plant cell extracts and for protein expressed from the cloned ACC synthase DNA. Based on the sequence alignment data from tomato, three peptides with largest stretches of amino acid sequence homology were selected.

(1) Peptide #1075, derived from the carboxy-terminus contained 35 amino acid residues as follows:

NVSPGSSFLCSEPGWFRVCFANMDNATLDVALNRI [SEQ ID NO:11]

(2) Peptide #1076, derived from the amino terminus contained 33 amino acids as follows:

YFDGWKAYDRDPYHSTKNSNGVIQMGLAENQLC [SEQ ID NO:12]

(3) Peptide #1077, from the middle region contained 38 amino acid residues as follows:

YSLSKDMGMPGFRVGIIYSYNDRVVSTARRMSSFGLVS [SEQ ID NO:13]

These peptides were used to immunize rabbits. A 1:1 emulsion of 200 µg/ml of peptide in complete Freund's Adjuvant was prepared, and 0.1 ml volumes were injected subcutaneously (sc) into three different rabbits at 17 to 18 sites on the animals' backs. Before injection, a preimmune serum sample was obtained. On day 19 after the first immunization, rabbits received two intramuscular (im) injections of 0.35 ml of a 1:1

emulsion of each peptide in incomplete Freund's adjuvant at 100 µg/ml. On day 35 after the first immunization, the day 19 im protocol was repeated. On day 92, each rabbit received a booster injection (im) with the same peptide emulsion as on day 19. Seven days later, the rabbits were bled, and serum was prepared.

Western blot analysis of antisera with the three peptides showed the presence of antibodies against each of the three peptides and strong signals indicating immunization was successful. Preimmune sera were negative.

Expression of Cloned ACC Synthase Genes *In Vitro*

Use was made of the ZAP Express Vector system which contains a bacteriophage T_3 promoter. Cloned ACC synthase genes are inserted by unidirectional *EcoRI/XhoI* site. The cloned insert can be excised from the phage in the form of a kanamycin-resistant pBK-CMV phagemid. The digestion of the phagemid from the three ACC synthase clones described above with *NotI* and *BamI* restriction enzymes showed the absence of these restriction sites in the inserts.

DNA from clones pPHSacc 41, pPHSacc 44 and pPHSacc49 and pRoseKacc7 was prepared, linearized with *NotI* and used for *in vitro* transcription. The reaction mixture (100 µl) contained Tris-HCl (pH7.9), 40mM; $MgCl_2$, 6mM; DTT, 10mM; spermidine, 2mM; m^7GpppG, 1mM; ATP, CTP and UTP, 0.5mM each; GTP, 25µM; Rnasin® (RNase inhibitor), 120 units; DNA template, 1-2 µg; and T_3 RNA polymerase, 50 units, as described in the inventor's publications. Samples were incubated at 37°C for 20 minutes. The GTP concentration in reaction mixture was raised to 0.5mM, and incubation was continued for one hour. Aliquots (3-5µl) of each reaction mixture were withdrawn and subjected to agarose gel (1.2%) electrophoresis to determine the quality and efficiency of transcript synthesis. The analysis of transcript showed expected size of RNA from each clone.

The *in vitro* transcripts from each clone were then translated at high efficiency using rabbit reticulocyte lysates as described by the present inventor (Ranu, R.S., *et al.* 1979, *Meth. Enzymol. 60*:459-484) except that they were made mRNA-dependent by treatment with micrococcal ribonuclease. The *in vitro* translation products were

immunoprecipitated with each of the three antisera described above or with a mixture of the antisera. The method used for immunoprecipitation and detection of ACC synthase protein was by Western blotting as described by the present inventor and colleagues in 1989 and recently published (Ranu, R.S., *et al,* (1996) *Gene Expression 5*:143-153). Translation products detected from each cloned ACC synthase gene was the size expected based on the size of the ORF of each clone. The *in vitro* translation product comigrate with the *in vivo* product upon gel electrophoresis.

The results described above are consistent with those obtained in other plant species indicating that the geranium ACC synthase genes belong to a multigene family. Despite the fact that these genes contain a termination codon immediately 5' to the initiation codon (in clones pPHSacc41 and pPHSacc44), or 21 nucleotides upstream from the initiation codon (clone pPHSacc49), the transcripts produced from these DNA sequences were translated efficiently *in vitro*. Clones pPHSacc44 and pPHSacc41 differ from each other in that for geranium, clone 44 contains an extra 780 bases of 3'UTR which each has a profound effect on translation of the transcript, reducing the translation dramatically. These results suggest a regulatory role for the 3'UTR in the expression of these genes.

Regeneration and Transformation

For the geranium, petioles from very young immature leaves from actively growing plants of Pelargonium hortorum cv Samba (sincerity could also have been used) were harvested and sterilized in 15% clorox for 15 minutes. They were then thoroughly rinsed with sterile distilled water (four times). The petioles were cut into 4-5mm segments and cultured on modified MS medium as further explained in *A Revised Medium For Rapid Growth And Bioassys With Tabacco Tissue Cultures,* Murashige T. and Skoog, F., Physiologia Plantarum 15,473-497 (1962). Modifications consist of one half concentration of major salts and pyridoxine HCl, 1mg/liter; nicotinic acid 1 mg/liter;

and thiamine HCl 10mg/liter. In addition, the medium used contained 5 μM BAP and 1 μM 1AA. After incubation of explants at 25°C in the dark for three days, they were transferred to light conditions. Regeneration became apparent by 15 days and continued for five weeks. The small shoots are then subcultured individually on MS medium containing 0.44 μM BAP and 0.11 μM IAA plus 400 mg/liter of L-glutamine. After about five weeks, they developed further to about 3-4cm long with 4-5 nodes. They were then subcultured on basal MS medium for rooting.

For transformation of geranium, pPHSacc41 was cut with *Not* I; the staggered ends were filled-in with dGTP and dCTP using Kelnow DNA polymerase The other end was cut with *Bam* HI for ligation into an agrobacterium binary vector in reverse orientation. The vector was prepared for ligation using *HPA* I and *Bam*I. The ligated vector (with PHSacc41 in reverse orientation) was used to transform agrobacterium tumefaciens 2760.

The petiole explants were cocultivated with agrobacterium for 5-10 minutes. After several days of cocultivation, agrobacterium cells were killed in the presence of cefotaxime (400 μg/ml) and Kananeycin (200 μg/ml). After about two weeks selection for transformants was continued. Ultimately, the transformed plants will be grown and tested for their various properties to determine which had successfully achieved the desired acc synthase modification. The selected genetically altered plants will be used to produce a new variety or line of plants wherein the alteration is stably transmitted from generation to generation.

The references cited above are all incorporated by reference herein, whether specifically stated as incorporated or not. Specifically, any references mentioned in the application for this patent as well as all references listed in any information disclosure originally filed with this or the priority application are hereby incorporated by reference in their entirety to the extent such may be deemed essential to support the enablement of the invention(s), however, applicant disclaims making or supporting any statements in said references which might be considered inconsistent with the patentability of the following claims or any aspect of the invention described.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation. While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This patent covers any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims. Further, it should be understood that various permutations and combination of the elements shown in the claims (whether method or apparatus) are possible and do fall within the scope of this disclosure.

DEPOSITS

The following illustrative plasmids encoding geranium ACC synthase were deposited at the American Type Culture Collection, Rockville, Maryland, prior to the filing date of the priority case of this patent under the requirements of the Budapest Treaty. These deposits were granted the following accession numbers and are hereby incorporated by reference:

1. pPHSacc41 cDNA clone comprising SEQ ID NO:1 - accession number ATCC 98177;

2. pPHSacc44 cDNA clone comprising SEQ ID NO:2 - accession number ATCC 98178; and

3. pPHSacc49 cDNA clone comprising SEQ ID NO:3 - accession number ATCC 98179.

Further, the following illustrative plasmid encoding rose ACC synthase has been deposited at the American Type Culture Collection, Rockville, Maryland under the requirements of the Budapest Treaty. This deposit has been granted the following accession number and is hereby incorporated by reference to the extent permissible:

1. pRoseKacc7 cDNA clone comprising SEQ ID NO:14 - accession number ATCC _____.

CLAIMS

WHAT IS CLAIMED IS:

1. An isolated DNA molecule encoding an ACC synthase enzyme of geranium which DNA molecule hybridizes with pPHSacc41 (SEQ ID NO:1), pPHSacc44 (SEQ ID NO:2), or pPHSacc49 (SEQ ID NO:3), or a functional derivative of said DNA molecule which hybridizes with SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3.

2. An isolated DNA molecule having substantial sequence homology with a molecule selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3.

3. An isolated DNA molecule according to claim 2 selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3.

4. An isolated DNA molecule encoding an ACC synthase enzyme of rose which DNA molecule hybridizes with pRoseKacc7 (SEQ ID NO:14), or a functional derivative of said DNA molecule which hybridizes with SEQ ID NO:14.

5. An isolated DNA molecule having substantial sequence homology with a molecule selected from the group consisting of SEQ ID NO:14.

6. An isolated DNA molecule according to claim 5 selected from the group consisting of SEQ ID NO:14.

7. An antisense oligonucleotide or polynucleotide encoding an RNA molecule which is complementary to at least a portion of an RNA transcript of the DNA molecule of any of claims 1-3, which RNA molecule hybridizes with said RNA transcript such that expression of said ACC synthase enzyme is altered.

8. An antisense oligonucleotide or polynucleotide according to claim 7 having between about six and about 100 nucleotides.

9. An antisense oligonucleotide or polynucleotide which is complementary to at least a portion of one strand of the nucleotide sequence SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3, or is complementary to at least a portion of an RNA sequence encoded by SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3.

10. An antisense oligonucleotide according to claim 9 which is complementary to at least a part of a 5' non-coding portion of one strand of the nucleotide sequence SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3.

11. An antisense oligonucleotide according to claim 7 which is complementary to at least a part of said nucleotide sequence SEQ ID NO:1, which part is:

(a) SEQ ID NO:1 nucleotides 1-50;
(b) SEQ ID NO:1 nucleotides 51-100;
(c) SEQ ID NO:1 nucleotides 101-150;
(d) SEQ ID NO:1 nucleotides 151-200;
(e) SEQ ID NO:1 nucleotides 201-250;

(f) SEQ ID NO:1 nucleotides 251-300;

(g) SEQ ID NO:1 nucleotides 301-350;

(h) SEQ ID NO:1 nucleotides 351-400;

(i) SEQ ID NO:1 nucleotides 401-450;

(j) SEQ ID NO:1 nucleotides 451-500; or

(k) any contiguous 50 nucleotides of SEQ ID NO:1 from nucleotide 1 to nucleotide 1945.

12. An antisense oligonucleotide according to claim 7 which is complementary to at least a part of said nucleotide sequence SEQ ID NO:2, which part is:

(a) SEQ ID NO:2 nucleotides 1-50;

(b) SEQ ID NO:2 nucleotides 51-100;

(c) SEQ ID NO:2 nucleotides 101-150;

(d) SEQ ID NO:2 nucleotides 151-200;

(e) SEQ ID NO:2 nucleotides 201-250;

(f) SEQ ID NO:2 nucleotides 251-300;

(g) SEQ ID NO:2 nucleotides 301-350;

(h) SEQ ID NO:2 nucleotides 351-400;

(i) SEQ ID NO:2 nucleotides 401-450;

(j) SEQ ID NO:2 nucleotides 451-500; and

(k) any contiguous 50 nucleotides of SEQ ID NO:2 from nucleotide 1 to nucleotide 2678.

13. An antisense oligonucleotide according to claim 7 which is complementary to at least a part of said nucleotide sequence SEQ ID NO:3, which part is:

(a) SEQ ID NO:3 nucleotides 1-50;

(b) SEQ ID NO:3 nucleotides 51-100;

(c) SEQ ID NO:3 nucleotides 101-150;

(d) SEQ ID NO:3 nucleotides 151-200;

(e) SEQ ID NO:3 nucleotides 201-250;

(f) SEQ ID NO:3 nucleotides 251-300;

(g) SEQ ID NO:3 nucleotides 301-350;

(h) SEQ ID NO:3 nucleotides 351-400;

(i) SEQ ID NO:3 nucleotides 401-450;

(j) SEQ ID NO:3 nucleotides 451-500; and

(k) any contiguous 50 nucleotides of SEQ ID NO:3 from nucleotide 1 to nucleotide 1878

14 An antisense oligonucleotide or polynucleotide encoding an RNA molecule which is complementary to at least a portion of an RNA transcript of the DNA molecule of any of claims 4-6, which RNA molecule hybridizes with said RNA transcript such that expression of said ACC synthase enzyme is altered.

15. An antisense oligonucleotide or polynucleotide according to claim 14 having between about six and about 100 nucleotides.

16. An antisense oligonucleotide or polynucleotide which is complementary to at least a portion of one strand of the nucleotide sequence SEQ ID NO:14, or is complementary to at least a portion of an RNA sequence encoded by SEQ ID NO:14.

17. An antisense oligonucleotide according to claim 16 which is complementary to at least a part of a 5' non-coding portion of one strand of the nucleotide sequence SEQ ID NO:14.

18. An antisense oligonucleotide according to claim 14 for which is complementary to at least a part of said nucleotide sequence SEQ ID NO:14, which part

is:

(a) SEQ ID NO:14 nucleotides 1-50;

(b) SEQ ID NO:14 nucleotides 51-100;

(c) SEQ ID NO:14 nucleotides 101-150;

(d) SEQ ID NO:14 nucleotides 151-200;

(c) SEQ ID NO:14 nucleotides 201-250;

(f) SEQ ID NO:14 nucleotides 251-300;

(g) SEQ ID NO:14 nucleotides 301-350;

(h) SEQ ID NO:14 nucleotides 351-400;

(i) SEQ ID NO:14 nucleotides 401-450;

(j) SEQ ID NO:14 nucleotides 451-500; or

(k) any contiguous 50 nucleotides of SEQ ID NO:14 from nucleotide 1 to nucleotide 1743.

19. A vector useful for transfection of a geranium plant cell, comprising:

(a) an oligonucleotide or polynucleotide according to claim 9;

(b) regulatory sequences required for expression of said oligonucleotide or polynucleotide in said cell.

20. A vector useful for transfection of a rose plant cell, comprising:

(a) an oligonucleotide or polynucleotide according to claim 16;

(b) regulatory sequences required for expression of said oligonucleotide or polynucleotide in said cell.

21. A vector according to claim 19 or 20, wherein said regulatory sequences comprise a promoter active in said cell.

22. A vector according to claim 21, wherein said regulatory sequences further comprise a polyadenylation signal.

23. A vector according to claim 21, wherein said promoter comprises a

heterologous promoter.

24. A vector according to claim 23, wherein said heterologous promoter is a viral promoter.

25. A vector according to claim 24, wherein said viral promoter is the CaMV 35S promoter or a promoter homologous to CaMV35S.

26. A vector according to claim 23, wherein said heterologous promoter is selected from the group consisting of the SSU gene promoter, ribulose bisphosphate carboxylase promoter, chlorophyll a/b binding protein promoter, potato ST-LS1 gene promoter, soybean heat shock protein hsp17.5-E promoter, soybean heat shock protein hsp17.3-B promoter, phenylalanine ammonia-lyase promoter, petunia 5-enolpyruvylshikimate-3-phosphate synthase gene promoter, *Rhizobium meliloti* FIXD gene promoter and nopaline synthase promoter.

27. A geranium cell transformed with a vector according to claim 19.

28. A mature geranium plant regenerated from a cell according to claim 27.

29. A plant part of a geranium plant according to claim 28.

30. A rose cell transformed with a vector according to claim 20.

31. A mature rose plant regenerated from a cell according to claim 30.

32. A plant part of a rose plant according to claim 31.

33. A method to alter expression of an ACC synthase enzyme in a geranium cell, plant or a cutting thereof, comprising

(a) transforming a geranium cell or plant with a vector according to claim 19; and

(b) allowing said antisense oligonucleotide or polynucleotide to be expressed

and to hybridize with nucleic acid molecules in said cell, plant or cutting which encode said ACC synthase enzyme, thereby altering said expression from said ACC synthase enzyme.

34. A method of producing a geranium plant having reduced ethylene production compared to an unmodified geranium plant, comprising the steps of:

(a) transforming a geranium plant with a vector according to claim 19;

(b) allowing the plant to grow to at least a plantlet stage;

(c) testing said plant for ACC synthase enzymatic activity or ethylene production;

(d) selecting a plant having altered ACC synthase activity and altered ethylene production compared to an unmodified geranium plant; and

then producing said plant.

35. A geranium plant produced according to the method of claim 34, or progeny, hybrids, clones or plants parts thereof, exhibiting reduced ACC synthase expression and reduced ethylene production.

36. A method for producing a geranium variety characterized by reduced expression or activity of an ACC synthase enzyme and reduced ethylene production compared to an unmodified geranium variety, comprising:

(a) producing a geranium plant in accordance with claim 34; and

(b) selfing said plant,

thereby generating said variety.

37. A method to alter expression of an ACC synthase enzyme in a rose cell, plant or a cutting thereof, comprising

(a) transforming a rose cell or plant with a vector according to claim 20; and

(b) allowing said antisense oligonucleotide or polynucleotide to be expressed and to hybridize with nucleic acid molecules in said cell, plant or cutting which encode said ACC synthase enzyme, thereby altering said expression from said ACC synthase enzyme.

38. A method of producing a rose plant having reduced ethylene production compared to an unmodified rose plant, comprising the steps of:

(a) transforming a rose plant with a vector according to claim 20;

(b) allowing the plant to grow to at least a plantlet stage;

(c) testing said plant for ACC synthase enzymatic activity or ethylene production;

(d) selecting a plant having altered ACC synthase activity and altered ethylene production compared to an unmodified rose plant; and

then producing said plant.

39. A rose plant produced according to the method of claim 38, or progeny, hybrids, clones or plants parts thereof, exhibiting reduced ACC synthase expression and reduced ethylene production.

40. A method for producing a rose variety characterized by reduced expression or activity of an ACC synthase enzyme and reduced ethylene production compared to an unmodified rose variety, comprising:

(a) producing a rose plant in accordance with claim 38; and

(b) selfing said plant,

thereby generating said variety.

41. A method for producing a variant plant of a non-geranium species, an ACC synthase gene of which is homologous to a geranium ACC synthase gene, in which variant plant the ACC synthase expression is altered in comparison to an unmodified plant of said species, comprising the steps of:

(a) identifying and isolating an ACC synthase gene of said species by hybridization with a DNA molecule according to claim 1 or 2;

(b) constructing a vector which comprises an antisense DNA sequence encoding at least a part of said gene identified in step (a) in an antisense orientation such that

(i) an RNA transcript of said antisense DNA sequence is complementary to said part of said gene, and

(ii) expression of said antisense DNA sequence alters expression of said ACC synthase gene;

(c) transforming a cell of a plant of said species with the vector of step (b) to generate a transformed cell; and

(d) regenerating a plant from said transformed cell of step (c);

thereby producing said variant plant.

42. A method for producing a plant variety in a non-geranium plant species characterized by reduced expression or activity of an ACC synthase enzyme and reduced ethylene production compared to a conventional variety of said species, comprising:

(a) producing a variant plant in accordance with claim 41; and

(b) selfing said plant

thereby generating said variety.

43. A method for genetically altering a plant, comprising the steps of:

(a) isolating mRNA of said plant using a 2-butoxyethanol precipitation technique with a critical mass amount of RNA for precipitation;

(b) constructing a cDNA library from said isolated mRNA;

(c) identifying and cloning a desired DNA sequence from said library;

(d) genetically altering said cloned DNA sequence; and

(e) transforming said plant with said altered DNA sequence,

thereby genetically altering said plant.

44. A method according to claim 43, wherein said step of isolating mRNA of said plant using a 2-butoxyethanol precipitation technique using a large amount of tissue from said plant comprises the step of using at least about 3 to 5 grams of tissue from said plant.

45. A method for genetically altering a plant, comprising the steps of:

(a) isolating mRNA of said plant using a 2-butoxyethanol precipitation

technique using a large amount of tissue from said plant;

(b) constructing a cDNA library from said isolated mRNA;

(c) identifying and cloning a desired DNA sequence from said library;

(d) genetically altering said cloned DNA sequence;

(e) transforming said plant with said altered DNA sequence,

thereby genetically altering said plant.

46. A method according to claim 43, wherein said plant is a member of a low RNA plant species.

47. A method according to claim 46, wherein said plant is a species of the genus *Pelargonium* or *Rosa*.

48. A method according to claim 43, 44, or 45 wherein said plant is selected from a group comprising woody plants.

49. A method according to claim 43, wherein said cloned DNA sequence encodes ACC synthase.

50. A method according to claim 49, wherein said plant is a geranium plant.

51. A method according to claim 50, wherein said cDNA is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3.

52. A method according to claim 49, wherein said plant is a rose plant.

53. A method according to claim 52, wherein said cDNA is selected from the group consisting of SEQ ID NO:14.

54. A method according to claim 43, wherein said isolating step (a) comprises the steps of:

(a) contacting said RNA with a binding partner for mRNA; and

(b) obtaining said bound mRNA.

55. A method for producing a genetically altered geranium plant, comprising the steps of:

(a) isolating geranium mRNA using a 2-butoxyethanol precipitation technique wherein at least about 3-5 grams of plant tissue starting material is used to attain a critical mass amount of RNA for precipitation;

(b) constructing a cDNA library from said isolated mRNA;

(c) identifying and cloning at least one DNA sequence from said library;

(d) genetically altering said cloned DNA sequence;

(e) transforming geranium cells with said altered DNA sequence; and

(f) regenerating said genetically altered geranium plant from said cells, which plant expresses said altered DNA sequence.

56. A method for producing a genetically altered rose plant, comprising the steps of:

(a) isolating rose mRNA using a 2-butoxyethanol precipitation technique wherein at least about 3-5 grams of plant tissue starting material is used to attain a critical mass amount of RNA for precipitation;

(b) constructing a cDNA library from said isolated mRNA;

(c) identifying and cloning at least one DNA sequence from said library;

(d) genetically altering said cloned DNA sequence;

(e) transforming rose cells with said altered DNA sequence; and

(f) regenerating said genetically altered rose plant from said cells, which

plant expresses said altered DNA sequence.

57. An isolated protein encoded by a DNA molecule according to claim 1, 2, or 3, or a functional derivative thereof.

58. An isolated protein having an amino acid sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, or a functional derivative thereof.

59. An isolated protein encoded by a DNA molecule according to claim 4, 5, or 6, or a functional derivative thereof.

60. An isolated protein having an amino acid sequence selected from the group consisting of SEQ ID NO:15, or a functional derivative thereof.

61. A method of isolating plant mRNA, comprising the steps of:

(a) extracting nucleic acids from sufficient plant tissue to attain a critical mass amount of RNA for precipitation;

(b) isolating RNA from said nucleic acids of step (a) using a 2-butoxyethanol precipitation technique;

(c) contacting said RNA with a binding partner for mRNA; and

(d) obtaining said bound mRNA;

thereby isolating said mRNA.

62. A method of isolating plant mRNA according to claim 61 wherein said binding partner is immobilized on a solid carrier thereby generating immobilized mRNA

and wherein said step of obtaining said bound mRNA comprises the step of eluting said mRNA from said carrier.

63. A method of isolating plant mRNA according to claim 62 wherein said step of extracting nucleic acids from sufficient plant tissue to attain a critical mass amount of RNA for precipitation comprises the step of using at least about 3 to 5 grams of tissue from said plant.

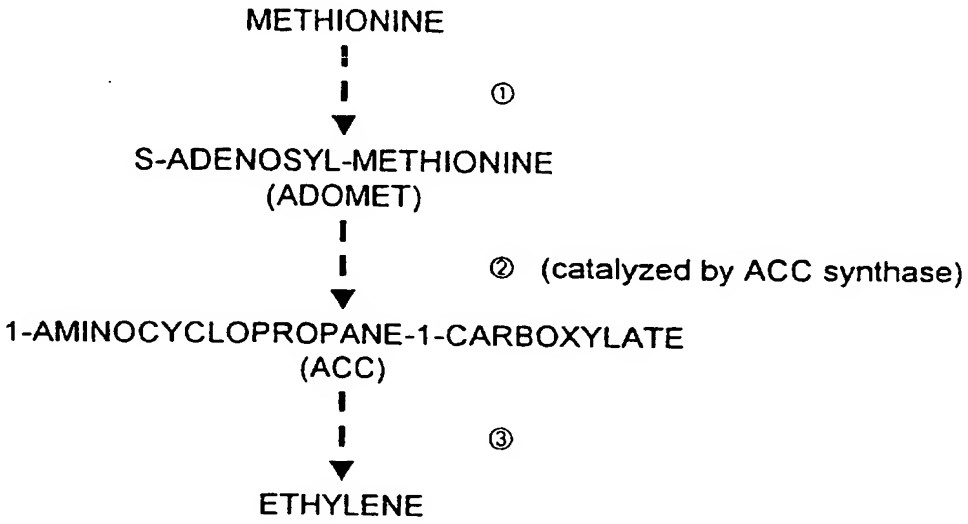

FIG 1

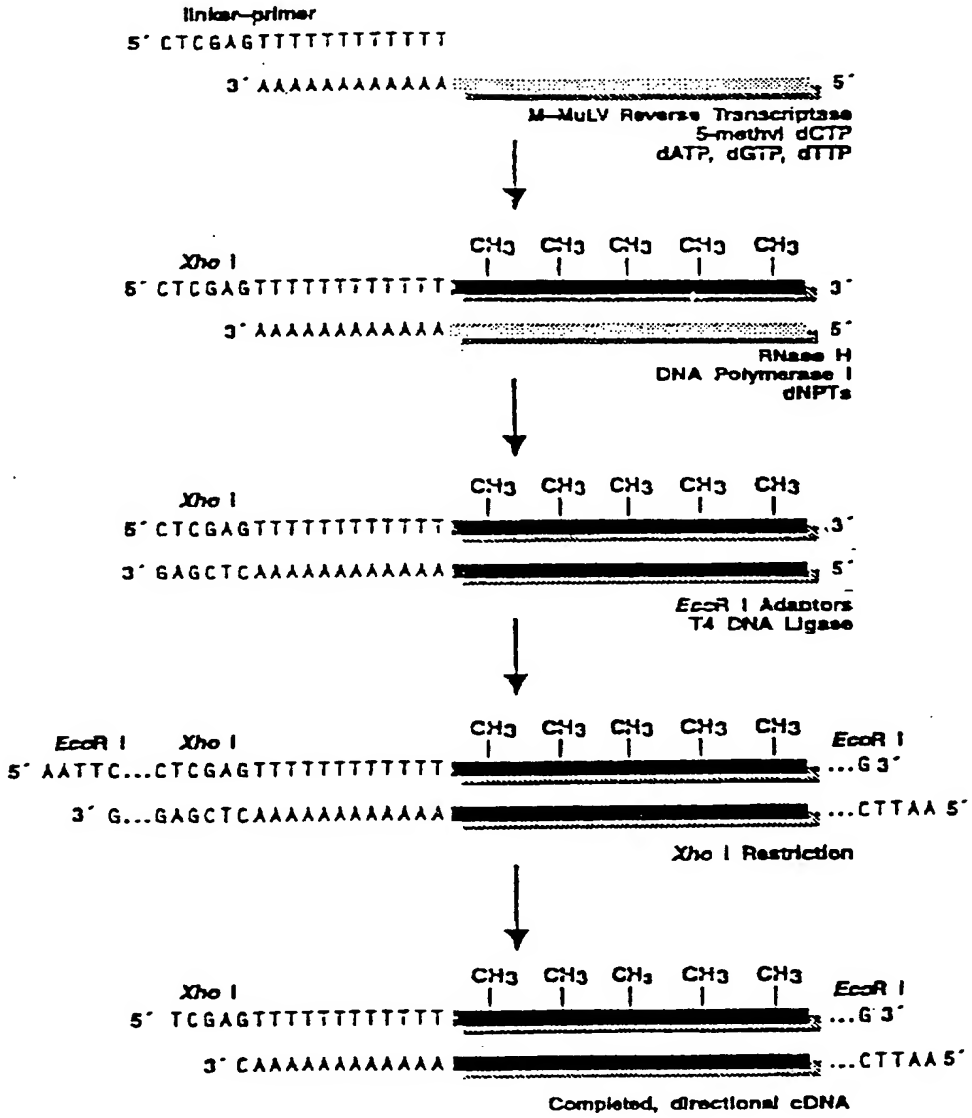

Fig 2 cDNA synthesis flow chart.

Plasmid pPHSacc41
[SEQ ID NO:1]

*GAATTCGGCACGAG*CTCGCTTCTGAGTGCCTAATTATTTTTGTCCAAGCTCTCAGTACGT
ACGTGTTGTACGTGTTTACATAG**ATG**GAGAACAAGAGCAAACAGCTTCTGTCAAAGATTG
CAACCAACGACGGACACGGCGAGAACTCCCCATATTTCGATGGTTGGAAGGCTTATGACC
GTGATCCGTTCCATCCGTCTCAGAATCCTAACGGTGTTATCCAG**ATG**GGTTTAGCTGAAA
ATCAGCTTTCATCTGACTTGATTGAAGATTGGGTGAGGTCCAACCCAGAAGCCTCAATCT
GCACTCTTGAGGGAGTTGGTAAGTTCAAGGACGTAGCTAACTTTCAGGACTACCATGGCC
TGCTGGAGTTCAGGCACGCCGTGGCTAAATTTATGAGCAGAGGAAGGGGCGGGAAGGTCA
CATTTGATCCCGACCGTGTCGTCATGAGCGGCGGACCGACCGGAGCCAACGAGCTCATCG
TCTTCTGTTTGGCCAATCCCGGCGACGCTTTCCTTCTCCCATCTCCTTATTATCCAGCAA
ACGACCGTGACTTGCAGTGGCGAACCGGAGCTCAGATCATTCCGGTGCACTGCAACAGCT
CCAACGGTTTCAAGATAACCAGAGAGGCACTAGAAAGATCATACGCACAAGCACAAGAAA
GCAACATAAACGTAAAAGGCGTGCTCTTAACCAACCCATCGAACCCTCTAGGCACAATTC
TGGACCGCGACACTCTCAAGAGCATCGTCAGCTTCGTCACCGACAACAACATCCACCTAG
TCATCGACGAAATCTACGCCGCCACCGTTTTCGCCGCCCCGGAGTTCGTAAGCGTCTCCG
AAATCCTCCAAGAAATGGACGACACCACGTGCAACCCCGACCTCATCCACATCGTGTACA
GCCTGTCCAAGGACTTGGGCATGCCCGGGTTCCGCGTCGGGATCGTGTACTCATTCAACG
ACGACGTCGTATCCTGCGCACGGAAGATGTCGAGCTTCGGGTTGGTGTCGACCCAGACGC
AGCACCTTCTCGCAGCGATGCTATCCGACGACGTTTTCGTGGAGCGGTTCCTCGCGGAGA
GCCGGAGCTTGGGGAGGAGGCACGGCGTGTTCACGAAAGGGCTCGAGGAGTTGGGGATTG
GGTGTTTAAAGAGCAACGCGGGGCTCTACTTCTGGATGGATTTGCGGAAGCTTCTAGAAG
AAGAGACGTTTGAGGCGGAGATGGTGCTGTGGAAGGTGATTATTAATGAGGTGAAGCTAA
ACGTGTCTCCGGGGTCGTCGTTTCATTGCGTGGAGCCGGGTTGGTTTAGGGTTTGCTTTG
CCAACATGGACGACGAGACGGTCCACGTGGCGCTGAAGAGGATCAGGGCGTTTGTGAGGA
AGAAGGAGGTGGGTCCGGTGAAGAGGAAGAGGTTCATGGACAACCTTAACCTCAGGCTGA
GCTTCTCGTCGCTAAGGTACGATGAGAGTGTGATGTTGTCGCCGCACATAATGGTGTCCC
CGCACTCGCCGCTTGTTCGTGCGAGAACA**TAA**TGAGCATGCACGTTTTTATTTGCTACTG
TTAGTAATTAACTAATTAATTGTTATTTGATTGTGTGCTGAATGTTGGATTCTTTCTTTG
TAGAAGTGAAGTATAGGAGATGTTTTTAACCAATTACCGTAGATATATATGCAGTGGAAT
TAAGAAAAATAAGAGGTTAAATATTAATTCCATGCATATATATGTAGGAAGGAATTGGTA
CATATTTTAGGGTTTGCTGATGTTTTCTTTCATCATGAATTGGTACATATTTATGATGTT
CAAGGCTCCAAGTGATGGATACATGGAGGATTCATTTGGATGCATGCCTTGCAAGAGTCA
GCAATCTTTGTTAATTAGTGTATGGTTTGTGAT**AATAAA**GATGCAAAATTCTGTGTTGTT
TTATTACTAAAAAAAAAAAAAAAAA

Figure 3
3/12

pPHSacc44

[SEQ NO:2]

GAATTCGGCACGAGTACGTGTTGTACGTGTTTACATAGATGGAGAACAAGAGCAAACAGCTTCTG
TCAAAGATTGCAACCAACGACGGACACGGCGAGAACTCCCCATATTTCGATGGTTGGAAGGCTTA
TGACCGTGATCCGTTCCATCCGTCTCAGAATCCTAACGGTGTTATCCAGATGGGTTTAGCTGAAA
ATCAGCTTTCATCTGACTTGATTGAAGATTGGGTGAGGTCCAACCCAGAAGCCTCAATCTGCACT
CTAGAGGGAGTTGGTAAGTTCAAGGACGTAGCTAACTTTCAGGACTACCATGGCCTGCTGGAGTT
CAGGCACGCCGTGGCTAAATTTATGAGCAGAGGAAGGGGCGGGAAGGTCACATTTGATCCCGACC
GTGTCGTCATGAGCGGCGGACCGACCGGAGCCAACGAGCTCATCGTCTTCTGTTTGGCCAATCCC
GGCGACGCTTTCCTTCTCCCATCTCCTTATTATCCAGGAAACGACCGTGACTTGCAGTGGCGAAC
CGGAGCTCAGATCATTCCGGTGCACTGCAACAGCTCCAACGGTTTCAAGATAACCAGAGAGGCCC
TAGAAAGATCATACGCACAAGCACAAGAAAGCAACATAAACGTAAAAGGCGTGCTCTTAACCAAC
CCATCGAACCCTCTAGGCACAATTCTGGACCGCGACACTCTCAAGAGCATCGTCAGCTTCGTCAC
CGACAACAACATCCACCTAGTCATCGACGAAATCTACGCCGCCACCGTTTTCGCCGCCCCGGAGT
TCGTAAGCGTCTCCGAAATCCTCCAAGAAATGGACGACACCACGTGCAACCCCGACCTCATCCAC
ATCGTGTACAGCCTGTCCAAGGACTTGGGCATGCCCGGGTTCCGCGTCGGGATCGTGTACTCATT
CAACGACGACGTCGTATCCTGCGCACGGAAGATGTCGAGCTTCGGGTTGGTGTCGACCCAGACGC
AGCACCTTCTCGCAGCGATGCTATCCGACGACGTTTTCGTGGAGCGGTTCCTCGCGGAGAGCCGG
AGCTTGGGGAGGAGGCACGGCGTGTTCACGAAAGGGCTCGAGGAGTTGGGGATTGGGTGTTTAAA
GAGCAACGCGGGGCTCTACTTCTGGATGGATTTGCGGAAGCTTCTAGAAGAAGAGACGTTTGAGG
CGGAGATGGTGCTGTGGAAGGTGATTATTAATGAGGTGAAGCTAAACGTGTCTCCGGGGTCGTCG
TTTCATTGCGTGGAGCCGGGTTGGTTTAGGGTTTGCTTTGCCAACATGGACGACGAGACGGTCCA
CGTGGCGCTGAAGAGGATCAGGGCGTTTGTGGGGAAGAAGGAGGTGGGTCCGGTGAAGAGGAAGA
GGTTCATGGACAACCTTAACCTCAGGCTGAGCTTCTCGTCGCTAAGGTACGATGAGAGTGTGATG
TTGTCGCCGCACATAATGGTGTCCCCGCACTCGCCGCTTGTTCGTGCGAGAACATAATGAGCATG
CACGTTTTTTTTTGCTACTGTTAGTAATTAACTAATTAATTGTTATTTGATTGTGTGCTGAATGT
TGGATTCTTTCTTTGTAGAAGTGAAGTATAGGAGATGTTTTTAACCAATTACCGTAGATATATAT
GCAGTGGAATTAAGAAAAATAAGAGGTTAAATATTAATTCCATGCATATATATGTAGGAAGGAAT
TGGTACATATTTTAGGGTTTGCTGATGTTTTCTTTCATCATGAATTGGTACATATTTATGATGTT
CAAGGCTCCAAGTGATGGATACATGGAGGATTCATTTGGATGCATGCCTTGCAAGAGTCAGCAAT
CTTTGTTAATTAGTGTATGGTTTGTGATAATAAAGATGCAAAATTCTGTGTTGTTTAAAAAAAAA
AAAAAAAAAAAAACTCGAGCAAATTGGAACCACCTTTCGATCCTTATGCAAACTCAATTAACTAC
CTCTTGGCTGCTTATTACATCCCTTATGTGGGACTTAATGGTTACGTTGGTACCACTCCAAATCT

Figure 4 (sheet 1)

TACCCGTACGGATTATAAAAGATTGGTGGCAGGACTGTTAGCTGTAGAGGGCGGACAGATGCTGA
TGCTGTTGTAAGAGCGTTCTGAGCGAGCGCGTTCAAGGTGTGAGCCGTACAATAAGACGGTGCTT
ACTTCACGGCGGCGATCTCGAAGCTGAGAAACAACCTTGGTCAAAATGGGATCAAAGATGAAGGG
ATATGGGTTCCCAAGTGCTTGAGCTGAGAATCGGACCCATAGTAACGTCTTATCGGCCGATCCCA
ACTCGTTGGGTACTCTAGGATGCCACCGGAGATATTGAGTATAATGTATACTACCGGAAATGAAA
GTACGGCCCGGTGGCTTCTTTCCCAAGGGTGCAAACGGCAAGATTGCTAGGTCTTATCTATTGAC
GAATAAATAATGTTTAATTTGAGTGTGCAAATAACAATCCAATAAGTTCGGAACTACTGGTTACA
TATTGCGTTGGTACATACTAGCTAACGTCTATGGGACTGAGTGGTTTGTGTAGTTTCAAACATTA
TTATGAGCTCGAGGTTGTATGATGGGAAATGTATTATATATGAAAGTTATTAATCAATTATAATG
TATACCGCTATATTCTTTTATGTAACTCTTATGA**AATAAA**GCAATAGTTCATAAAAAAAAAAAAA
AAAAAAAAAAAAA

Figure 4 (sheet 2)

pPHSacc49
[SEQ ID NO:3]

*GAATTCGGCACGAG*CTTCACAGCACAGCTCTTTAAGCAACCATCATCATCTTTTG
CATATTAATTCTGAGGATTTTCTTTGAGCAAAACAACATCGATCAAAA**ATG**GTGA
ACATGTCCTCAACAACTAACCAAAGAACATTGTTATCTAAGATGGCAACTGGAGA
TGGACATGGCGAAAACTCACCTTACTTTGATGGCTGGAAAGCTTACGACAACAAT
CCTTTCCATCTCACCCAAAACCCTCAAGGTGTCATCCAGATGGGCCTCGCAGAAA
ATCAGCTTTCTTTCGAGTTGATTGAGCAATGGGTCCTTAACAACCCACAAGCCTC
CATTTGCACAGCACAAGGTCTGCAAGAATTCAAGGACACTGCAATCTTTCAAGAT
TACCATGGCTTGCAGAGTTCAGATATGCTGTTTGCAAATTTCATGGGAAAGGTGA
GAGGAAACAGAGTAACATTTAACCCAGATCGCATAGTTATGAGTGGAGGAGCAAC
TGGAGCTCATGAAATGATTGCCTTCTGTTTGGCTGATCCTGGCGATGCTTTTCTT
GTCCCAACTCCTTATTATCCTGGATTTGATAGAGACCTGAGGTGGAGAACTGGTG
TGCAGCTAATTCCTGTAGTAGTCTGTGAAAGTGAAAACAATTTCAGGATCACCCG
AAGTGCCTTAGAAGAAGCCTATGAGAGAGCTCAAGAGGACAAGATTAGAGTGAAG
GGATTGCTCATAACAAACCCATCAAACCCACTAGGAACTATCCTGGACAGAGAGA
CACTAGTCAGTCTAGTGAGCTTCATCAATGAAAAGAACATTCACTTGGTCTGTGA
TGAAATCTACGCCGCCACAGTCTTCTCTCAGCCCGCTTTCGTTAGCATTGCTGAG
GTTATCGAGCAAGAGAACGTTTCGTGCAACCGCGACCTCATCCACATTGTCTACA
GCCTGTCCAAGGACATGGGCTTCCCTGGCTTCAGGGTGGGGATTGTCTACTCCTA
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CATTCATCGTGGAGAGCGCGAAGAGGCTAGCGAGAAGGTACACAACCTTCACAAG
AGGGCTTGCACAAGTGAACATTGGATGCCTAAAGAGCAATGGGGGGTTATTCATA
TGGATGGACTTGAGGAGGCTTCTCAAGGAGAAGACTTTCGAGGCGGAGATGGCTC
TGTGGAGAGTGATAATCAATGAAGTGAAGCTAAATGTGTCGCCAGGGGCGTCGTT
CCATTGCTCGGAGCCAGGGTGGTTTAGAGTGTGCTTTGCCAACATGGATGACTTG
ACGATGCAGGTGGCTCTGAGGAGGATCATAACATTTGCACTTCAGAACAAGGAAG
CTGCGGTTTTGCCTGCAATCAAGAGACAGTGTTGGCAAAACAACCTTGGAAGGCT
CAGCTTGTCTTTCAGGAGATTTGATGATTTCACGATGTCTCCAATGTCCCCTCAC
TCCCCAATACAATCACCACTTGTGAGAGCCACT**TAG**AAACACATGAATAATAGAG
AATAACGGGCGATGCGGCCGCCAAAAATAGGTTGATCTATGTATGCATTAACGTT
TTTAGTTAATCTGTGTTTAATAGTATAACAAGAAGGAACAAAATGTATTCTTTCT
GTATAAATAACCCGGGGTAGGTTGATCTATGTATGCATTAACGTTTTTAGTTAAT
CTGTGTTTATATGTATAACAAGAAGGAACAAAATGTATTCTTTCTGTAT**AAATAA**
CCCAAACTTAGAAGATGCTTGCTGTGCATCCTTCTGGGAAAAAAAAAAAAAAAAA
AAAAAAAA

Figure 5

Deduced Amino Acid Sequence encoded by pPHSacc41

[SEQ ID NO:4]

MENKSKQLLSKIATNDGHGENSPYFDGWKAYDRDPFHPSQNPNGVIQMGL
AENQLSSDLIEDWVRSNPEASICTLEGVGKFKDVANFQDYHGLLEFRHAV
AKFMSRGRGGKVTFDPDRVVMSGGPTGANELIVFCLANPGDAFLLPSPYY
PANDRDLQWRTGAQIIPVHCNSSNGFKITREALERSYAQAQESNINVKGV
LLTNPSNPLGTILDRDTLKSIVSFVTDNNIHLVIDEIYAATVFAAPEFVS
VSEILQEMDDTTCNPDLIHIVYSLSKDLGMPGFRVGIVYSFNDDVVSCAR
KMSSFGLVSTQTQHLLAAMLSDDVFVERFLAESRSLGRRHGVFTKGLEEL
GIGCLKSNAGLYFWMDLRKLLEEETFEAEMVLWKVIINEVKLNVSPGSSF
HCVEPGWFRVCFANMDDETVHVALKRIRAFVRKKEVGPVKRKRFMDNLNL
RLSFSSLRYDESVMLSPHIMVSPHSPLVRART

Figure 6

Deduced Amino Acid Sequence encoded by pPHSacc44

[SEQ ID NO:5]

MENKSKQLLSKIATNDGHGENSPYFDGWKAYDRDPFHPSQNPNGVIQMGLAENQLSSDLI
EDWVRSNPEASICTLEGVGKFKDVANFQDYHGLLEFRHAVAKFMSRGRGGKVTFDPDRVV
MSGGPTGANELIVFCLANPGDAFLLPSPYYPGNDRDLQWRTGAQIIPVHCNSSNGFKITR
EALERSYAQAQESNINVKGVLLTNPSNPLGTILDRDTLKSIVSFVTDNNIHLVIDEIYAA
TVFAAPEFVSVSEILQEMDDTTCNPDLIHIVYSLSKDLGMPGFRVGIVYSFNDDVVSCAR
KMSSFGLVSTQTQHLLAAMLSDDVFVERFLAESRSLGRRHGVFTKGLEELGIGCLKSNAG
LYFWMDLRKLLEEETFEAEMVLWKVIINEVKLNVSPGSSFHCVEPGWFRVCFANMDDETV
HVALKRIRAFVGKKEVGPVKRKRFMDNLNLRLSFSSLRYDESVMLSPHIMVSPHSPLVRA
RT

Figure 7

Deduced Amino Acid Sequence encoded by pPHSacc49

[SEQ ID NO:6]

MVNMSSTTNQRTLLSKMATGDGHGENSPYFDGWKAYDNNPFHLTQNPQGVIQMGL
AENQLSFELIEQWVLNNPQASICTAQGLQEFKDTAIFQDYHGLQSSDMLFANFMG
KVRGNRVTFNPDRIVMSGGATGAHEMIAFCLADPGDAFLVPTPYYPGFDRDLRWR
TGVQLIPVVVCESENNFRITRSALEEAYERAQEDKIRVKGLLITNPSNPLGTILD
RETLVSLVSFINEKNIHLVCDEIYAATVFSQPAFVSIAEVIEQENVSCNRDLIHI
VYSLSKDMGFPGFRVGIVYSYNDAVVNCARKMSSFGLVSTQTQHLIASMLSDDEF
VDTFIVESAKRLARRYTTFTRGLAQVNIGCLKSNGGLFIWMDLRRLLKEKTFEAE
MALWRVIINEVKLNVSPGASFHCSEPGWFRVCFANMDDLTMQVALRRIITFALQN
KEAAVLPAIKRQCWQNNLGRLSLSFRRFDDFTMSPMSPHSPIQSPLVRAT

Figure 8

pRoseKacc7
[SEQ ID. NO:14]

GCCTTGGCTTTCCTCCCTTCGCTTTCTTCTTCTTCTTCTTCATCATCGTACTCTCCGACG

ACCCGAAACCCCACCGCGACCCGGCCCGGATGTCTCCAATATGACCCGGACCCGAGACGA

AGACCGGCGACCCAGCAGCAGCAGCAGCGGCGGCGGAGGAGGCGCCGATGAGAGTTATAG

TCCCTCTACAAGGCGTGGTTCAAGGCAGAGGAGGACTCGTTCTCGGCTCCGTCATACCAT

GCGCGCTCTTCTATTTCCTCCAGCTTTATC**ATG**AAACGTCACCGTTCCAACTCCAACCCG

CCGACTCCGCCGCCTTCTCCGGACTCGGACTCGGACCACCACCCCGCCGGGCAGTTGGTG

GAAGTTCCGGTTCTGCCCCGGTCGATGTCGAGGTCCCATCTCTCTCCGAGGAACCCGGGT

CCGGTACATGTCTCGGGTCGGGCCAATTCGGTTTTGAAAGGCGGTGAGCCGCCGTATTAT

GTCGGCTTGAGGAAGGTGGCGGAGGATCCGTACGACGAGTTGGGTAACCCGGATGGGGTT

ATTCAGCTGGGTTTGGATGAAAACAAGTTAGCTTTGGACTTGGTTCGAGATTGGCTACTG

GAGAATGCAAAGGATGCAATACTGGGTGGTGAGGAGCTTGGGATTAGTGGGATTGCTTGT

TACCAGCCTTCTGATGGTTTAATGGAGCTCAAACTGGCTGTGGCAGGATTCATGTCTAAG

GCCATCGGAAATTCAGTTACGTACAACCCCTCACAAATTGTATTGACAGCTGGTGCAACC

CCTGCAATTGAGATTCTAAGCTTCTGCCTAGCAGACAGTGGAAACGCATTTCTCGTTCCG

GCACCATATTACCCTGGTTTGGACAGAGATGTGAAGTGGCGAACTGGAGTGGAGATAATA

CCTGTTCCATGCCGCAGTGCTGACAAATTCAATTTAAGTATAACTGCACTTGATCGAGCA

TTCAACCAGGCAAAGAAACGTGGTGTAAAAGTTCGTGGGATTATAATTTCAAATCCTTCA

AATCCTGGTGGCAGTTTACTTACTCGTGAATCACTTTACAACCTTCTGGACTTTGCCCGA

GAGAAGAACATTCATATAATCTCAAATGAATTGTTTGCTGGATCCACGTATGGAAGTGAA

GAGTTTGTTAGCATGGCAGAAATCGTTGATTTGGAAGATCTCGACCAGAACAGAGTGCAT

ATAGTATATGGCATATCGAAAGATCTCTCACTTCCAGGTTTCAGGGTGGGTGCCATCTAC

TCCTTTAACAAGAATGTCTTGACTGCTGCTAAAAAGTTGACAAGGTTCTCTTCTATCTCC

GCCCCATCCCAACGGTTGCTTATCTCTATGCTTTCAGACACCAAATTTATGCATAAGTTC

ATCGAGATTAACAGAGAAAGGCTCCGTGGAATGTATCTTAGATTTGTGACAGGATTGAAG

Figure 9 (sheet 1)

CAATTGGGCATTGAGTGCACAAAGAGCAATGGGGGTTTCTACTGTTGGGCAGACTTGAGT

GGGTTAATTCGCTCTTACAGTGAGAAAGGGGAGCTTGAGCTCTGGGATAGGTTGTTGAAT

GTAGGTAAGCTCAATGTTACTCCTGGATCTTCTTGTCATTGTATTGAACCGGGATGGTTC

CGGTTTTGTTTTACGACGTTGACTGAAAAAGATATCCCTGTTGTTATAGAACGAATTCGG

AATATTGCCGAAACATGTAAATCACACAGTTGAAATGTTCGTTCATTCTACTCAAAAAAA

AAA

Figure 9 (sheet 2)

Deduced Amino Acid Sequence encoded by pRoseKacc7 [SEQ ID. NO:15]

MKRHRSNSNPPTPPPSPDSDSDHHPAGQLVEVPVLPRSMSRSHLSPRNPGPVHV
SGRANSVLKGGEPPYYVGLRKVAEDPYDELGNPDGVIQLGLDENKLALDLVRDW
LLENAKDAILGGEELGISGIACYQPSDGLMELKLAVAGFMSKAIGNSVTYNPSQ
IVLTAGATPAIEILSFCLADSGNAFLVPAPYYPGLDRDVKWRTGVEIIPVPCRS
ADKFNLSITALDRAFNQAKKRGVKVRGIIISNPSNPGGSLLTRESLYNLLDFAR
EKNIHIISNELFAGSTYGSEEFVSMAEIVDLEDLDQNRVHIVYGISKDLSLPGF
RVGAIYSFNKNVLTAAKKLTRFSSISAPSQRLLISMLSDTKFMHKFIEINRERL
RGMYLRFVTGLKQLGIECTKSNGGFYCWADLSGLIRSYSEKGELELWDRLLNVG
KLNVTPGSSCHCIEPGWFRFCFTTLTEKDIPVVIERIRNIAETCKSHS

Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17644

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04
US CL :536/23.6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAN DER STRAETEN et al. Cloning and Sequence of Two Different cDNAs Encoding 1-Aminocyclopropane-1-Carboxylate Synthase in Tomato. Proceedings of the National Academy of Sciences USA. June 1990, Vol. 87, pages 4859-4863, especially page 4861, Figure 2).	1-2
X	NAKAJIMA et al. Molecular Cloning and Sequence of a Complementary DNA Encoding 1-Aminocyclopropane-1-Carboxylate Synthase Induced by Tissue Wounding. Plant Cell Physiology. 07 September 1990, Vol. 31, No. 7, pages 1021-1029, especially page 1025, Figure 3.	1-2

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 JANUARY 1998	Date of mailing of the international search report 10 FEB 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer THANDA WAI Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17644

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SATO et al. The 1-Aminocyclopropane-1-Carboxylate Synthase of Cucurbita: Purification, Properties, Expression in Escherichia coli and Primary Structure Determination by DNA Sequence Analysis. The Journal of Biological Chemistry. 25 February 1991, Vol. 266, No. 6., pages 3752-3759, especially page 3755, Figure 6).	1-2
X	BOTELLA et al. Identification and Characterization of a Full-Length cDNA Encoding for an Auxin-Induced 1-Aminocyclopropane-1-Carboxylate Synthase from Etiolated Mung Bean Hypocotyl Segments and Expression of Its mRNA in Response to Indole-3-Acetic Acid. Plant Molecular Biology. 05 November 1992, Vol 20, pages 425-436, especially page 430, Figure 1.	1-2
X	LIANG et al. The 1-Aminocyclopropane-1-Carboxylate Synthase Gene Family of Arabidopsis thaliana. Proceedings of the National Academy of Sciences USA. 20 November 1992, Vol. 89, pages 11046-11050, especially page 11048, Figure 3.	1-2
X	BAILEY et al. Nucleotide Sequence of the Nicotiana tabacum cv Xanthi Gene Encoding 1-Aminocyclopropane-1-Carboxylate Synthase. Plant Physiology. 18 November 1992, Vol. 100, pages 1615-1616, especially 1615, Figure 1.	1-2
A	US 5,416,250 A (FERRO et al.) 16 May 1995, column 1, line 13 to column 3, line 51.	1-3
A	ABELES et al. 'The Biosynthesis of Ethylene.' In: Ethylene in Plant Biology, second edition. San Diego: Academic Press, Inc., 1992, pages 26-54.	1-3
A	NADEAU et al. Temporal and Spatial Regulation of 1-Aminocyclopropane-1-Carboxylate Oxidase in the Pollination-Induced Senescence of Orchid Flowers. Plant Physiology. September 1993, Vol. 103, pages 31-39, especially pages 31-32.	1-3
X	WANG et al. A Flower Senescence-Related mRNA from Carnation Shares Sequence Similarity with Fruit Ripening-Related mRNAs Involved in Ethylene Biosynthesis. Plant Physiology. July 1991, Vol. 96, pages 1000-1001, especially page 1000.	1-3
A	REID et al. Ethylene and Flower Senescence. Plant Growth Regulation. January 1992, Vol. 11, pages 37-43, see entire document.	1-3

INTERNATIONAL SEARCH REPORT

International application No.
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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17644

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, AGRICOLA, BIOSIS, EMBASE, WPIDS, and MPSEARCH (for SEQ ID NO:1, 2, and 3)

search terms: aminocyclopropane, carboxylate synthase, ACC synthase, sequenc?

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1-3, drawn to an isolated DNA molecule encoding an ACC synthase enzyme of geranium, the first product.

Group II, claims 4-6, drawn to an isolated DNA molecule encoding an ACC synthase enzyme of rose, the second product.

Group III, claims 7-13, 19, 21-29, and 33-36, drawn to antisense oligonucleotides of the geranium ACC synthase enzyme of Group I, vectors comprising such oligonucleotides, and to a method of altering ACC synthase enzyme in a geranium cell, plant, or cutting by transformation with such vectors, the third product and method of using the product.

Group IV, claims 14-18, 20-26, 30-32, and 37-40, drawn to antisense oligonucleotides of the rose ACC synthase enzyme of Group I, vectors comprising such oligonucleotides, and to a method of altering ACC synthase enzyme in a geranium cell, plant, or cutting by transformation with such vectors, the fourth product and method of using the product.

Group V, claims 41-42, drawn to a method for producing a variant plant of a non-geranium species by transforming with an ACC synthase gene which is homologous to the geranium ACC synthase gene.

Group VI, claims 43-56 and 61-63, drawn to a method of genetically altering a plant by transforming with cDNAs made from mRNA isolated using a 2-butoxyethanol precipitation technique.

Group VII, claim 57-59, drawn to an isolated protein encoded by a DNA molecule encoding the geranium ACC synthase enzyme of Group I.

Group VIII, claim 60, drawn to an isolated protein encoded by a DNA molecule encoding the rose ACC synthase enzyme of Group II.

The inventions listed as Groups I-VIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the products of Groups I, II, III, IV, VII, and VIII are distinct. The methods of Groups V and VI do not utilize the product of Group I. PCT rule 13 does not provide for multiple products or multiple methods of using within a single application (37 CFR 1.475(d)).